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CELL-METABOLISM-D-18-01145 revision

# Tumor-induced cholesterol efflux from macrophages drives IL-4 mediated reprogramming and tumor progression.

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**Running title:** Cholesterol efflux from TAM drives tumor progression

## Highlights

- Monocyte-derived TAM gradually replace resident peritoneal macrophages in metastatic ovarian cancer
- Ovarian cancer cells promote membrane-cholesterol efflux in TAM
- Cholesterol-efflux depletes lipid rafts and increases IL-4 signaling in TAM
- Inhibition of ABC transporters reverts the tumor-promoting functions of TAM in ovarian cancer

## eTOC blurb

Goossens et al. show that cancer cells scavenge membrane cholesterol from macrophages in tumors which reprogrammes them towards an immune-suppressive and tumor-promoting phenotype and makes them resistant to activation by anti-tumor cytokines.

## Summary

Tumor-associated macrophages (TAM) have been shown to have important roles in the malignant progression of various cancers. However, macrophages also possess intrinsic tumoricidal activity, but rapidly adopt an alternative phenotype within tumors associated with immune-suppression and trophic functions supporting tumor growth. The mechanisms that promote TAM polarization remain poorly understood, these mechanisms may represent important therapeutic targets to block the tumor-promoting functions of TAM and restore their anti-tumor potential. Here we have characterized TAM in a mouse model of metastatic ovarian cancer. We show that ovarian cancer cells promote membrane-cholesterol efflux and depletion of lipid rafts from macrophages. Increased cholesterol efflux promoted IL-4 mediated reprogramming while inhibiting IFN $\gamma$ -induced gene expression. These studies reveal an unexpected role for membrane-cholesterol efflux in driving the tumor-promoting functions of TAM, while rendering them refractory to pro-inflammatory stimuli. Thus, preventing cholesterol efflux in TAM may represent a novel therapeutic strategy to block pro-tumor functions and restore anti-tumor immunity.

## Keywords

Tumor-associated macrophages, Ovarian cancer, Cholesterol efflux, Lipid rafts, IL-4 signaling

## Introduction

There is now a wealth of clinical and experimental evidence that strongly links tumor-associated macrophages (TAM) with tumor progression, invasion and metastasis (Noy and Pollard, 2014). In the vast majority of published studies, increased numbers of TAM correlate with poor prognosis, but in some cases, specific TAM subsets have been associated with beneficial outcomes (de Vos van Steenwijk et al., 2013; Ino et al., 2013). Indeed, macrophages have been shown to possess intrinsic tumoricidal activity and promote the activation of cytotoxic lymphocytes (Bonnotte et al., 2001; Hagemann et al., 2008; Mytar et al., 1999), but they rapidly adopt an alternative phenotype within tumors, associated with immune-suppression and trophic functions that support tumor growth (Mantovani et al., 2008). However, the mechanisms that promote TAM reprogramming in the tumor-microenvironment remain poorly understood.

In mammals, macrophages are found in all tissues after birth and are endowed with trophic functions that contribute to organ development and remodelling (Pollard, 2009). Recent advances in genetic fate-mapping techniques have revealed that the majority of tissue-resident macrophages, at least in steady-state, develop from embryonic precursors and are maintained by local proliferation with little input from hematopoietic stem cells (HSC) in the bone marrow (Schulz et al., 2012). Subsequent studies have shown that embryonic macrophages can be gradually replaced by HSC-derived blood monocytes, to varying degrees depending on the specific context (Ginhoux and Guillemin, 2016). But the functional implications of these distinct developmental origins and certainly their respective contributions to tumor progression remain unclear. In a recent study, both tissue-resident macrophages of embryonic origin and monocyte-derived TAM were shown to contribute towards tumor growth in a mouse model of pancreatic cancer (Zhu et al., 2017).

The phenotype of tissue-resident macrophages is dictated by the tissue-specific signals in their respective niche (Gosselin et al., 2014; Lavin et al., 2014). However, during inflammation or tissue stress, monocyte-derived macrophages can be recruited into tissues and their functional reprogramming is dictated by the pathological context. It is now widely appreciated that macrophages follow a multi-dimensional model of activation states with distinct phenotypic and functional properties in response to different stimuli in the tissue microenvironment and can maintain considerable plasticity (Murray et al., 2014; Xue et al., 2014). Along these lines, TAM in various experimental models and human cancers have been shown to express unique sets of gene patterns including the production of specific chemokines, cytokines and growth factors linked with tumor progression, such as CCL2, TNF, VEGF, basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMPs) (Kratovich et al., 2015). Nevertheless, TAM are invariably reprogrammed towards a functional state that supports tumor growth and



immune-suppression and away from inflammatory phenotypes that could be associated with anti-tumor functions. The specific mechanisms that drive TAM accumulation and polarization in different tumors remain unclear. Several studies have shown that TAM are CSF-1 dependent, as are most tissue macrophages, and CSF-1 signaling has been suggested to be an important factor in their reprogramming towards pro-tumor functions (Martinez et al., 2006; Noy and Pollard, 2014). CSF-1 and IL-4 signaling in TAM was later shown to cooperatively promote growth of lung metastases in the MMTV-pyMT mouse model of mammary carcinogenesis (DeNardo et al., 2009). However, although primary tumor-development in this model was CSF-1 dependent (Lin et al., 2006), IL-4 signaling in TAM did not impact primary tumors (DeNardo et al., 2009). Subsequent studies showed that the development of lung metastases, but not primary tumors, in the same model critically requires the recruitment of CCR2-dependent monocytes (Qian et al., 2011). Suggesting that IL-4 signaling, specifically in monocyte-derived TAM, promotes metastatic disease in this model.

Here we have characterized TAM in a mouse model of metastatic ovarian cancer. We show that monocyte-derived TAM gradually replaced resident macrophages in this model and displayed an upregulation of cholesterol metabolism and reverse cholesterol efflux pathways during tumor progression. Further experiments revealed that ovarian cancer cells actively promoted plasma membrane cholesterol efflux from macrophages and the subsequent loss of cholesterol-rich membrane microdomains, or so called “lipid rafts”. Increased cholesterol efflux promoted enhanced IL-4 signaling in macrophages while inhibiting IFN $\gamma$ -induced gene expression. IL-4 induced gene expression in TAM is associated with tumor-promoting functions, including increased arginine metabolism promoting immune-suppression and trophic functions, supporting invasion and metastasis. Whereas, IFN $\gamma$  induced gene expression in TAM drives anti-tumor functions. We further demonstrate that IL-4 signaling and cholesterol efflux pathways in TAM significantly contribute to tumor progression *in vivo*. These studies suggest an important role for membrane-cholesterol efflux in driving IL-4 signaling and tumor-promoting functions of TAM in ovarian cancer, while rendering them refractory to reprogramming by anti-tumor cytokines. Therefore, preventing cholesterol efflux in TAM could represent a novel therapeutic strategy to block pro-tumor functions and restore anti-tumor immunity.

## Results

### ***Origins of TAM during ID8 tumor development.***

High grade serous ovarian cancer (HGSC) is frequently associated with colonisation of the peritoneal cavity by cancer cells (George et al., 2016). ID8 cells are spontaneously transformed mouse ovarian surface epithelial cells (Urzua et al., 2016), when adoptively transferred by intra-peritoneal (i.p.) injection in syngeneic mice, these cells progressively develop a malignant ascites with tumor nodules throughout the peritoneal cavity (Hagemann et al., 2008), which is characteristic of HGSC. The peritoneal cavity is populated by two major subsets of serosal macrophages; large peritoneal macrophages (LPM), which are most abundant, and a minor population of small peritoneal macrophages (SPM) (Ghosn et al., 2010). Previous studies have shown that SPM and LPM have distinct developmental origins; SPM develop from blood monocytes which are derived from bone marrow progenitors, whereas LPM are derived from embryonic progenitors and are maintained independently of blood monocytes, retaining proliferative capacity for self-renewal (Yona et al., 2013). More recent studies have shown that LPM can be progressively replaced by long-lived bone marrow-derived macrophages that maintain self-renewal potential (Bain et al., 2016). To monitor the dynamics of peritoneal macrophages (PM) during ID8 tumor growth, we first characterized macrophage subsets by flow cytometry. SPM and LPM can be distinguished by F4/80 and MHCII expression; SPM are MHCII<sup>hi</sup> F4/80<sup>lo</sup> whereas LPM are F4/80<sup>hi</sup> MHCII<sup>lo</sup> (Fig.1A). LPM represent approximately 80 % of PM in naïve mice, however, after seeding of ID8 cells in the peritoneal cavity, a significant population of F4/80<sup>int</sup> MHCII<sup>int</sup> PM rapidly accumulates (intPM; Fig.1A). Kinetic analysis of total cell numbers revealed that LPM numbers remain relatively constant throughout tumor progression, while intPM progressively accumulate and eventually become the dominant TAM population (Fig.1B).

To determine the dynamics of PM subsets during ID8 tumor growth, we performed fate-mapping studies with shielded radiation chimera mice. Radiation chimeras can be used to determine the contribution of bone marrow-derived progenitors towards cells in a given tissue. However, irradiation kills tissue-resident macrophages that then become replaced by monocyte-derived cells, thus to distinguish tissue-resident cells from monocyte-derived macrophages from the bone marrow, it is necessary to protect the tissue from the effects of radiation using lead shielding. To study the origins of PM subsets during ID8 tumor growth, we shielded the abdomen of host C57BL6 CD45.1 congenic mice during irradiation and then adoptively transferred a mixture of bone marrow cells from mice expressing both CD45.1 and CD45.2 (CD45.1/2) and *Ccr2*<sup>-/-</sup> mice, expressing only CD45.2. This allowed the distinction between host (CD45.1) and donor cells (CD45.1/2 or CD45.2), as well as their CCR2-dependency, CD45.2 single-positive cells being CCR2-dependent. Due to the low engraftment efficiency of *Ccr2*<sup>-/-</sup> bone marrow cells, *Ccr2*<sup>-/-</sup> donor cells were mixed at a ratio of 4:1 with

competitor B6.CD45.1/2 cells. Five weeks after bone marrow engraftment, chimeric mice were injected with ID8 cells to track the contribution of bone marrow-derived cells to PM subsets (Fig.1C). CD45.1 and CD45.2 expression in TAM subsets was measured by flow cytometry a further 8 weeks after injection of ID8 cells, a total of 13 weeks after adoptive transfer of bone marrow cells, chimerism was normalised to blood monocytes. These experiments revealed that SPM and intPM were derived from CCR2-dependent bone marrow progenitors, with almost 100 % chimerism after 8 weeks of tumor growth (Fig.1D,E). However, at this time point, LPM only showed approximately 30 % chimerism, implying that LPM are more gradually replaced by bone marrow-derived cells during tumor development. To confirm the CCR2-dependency of intPM, we analyzed the accumulation of PM subsets in full CCR2 deficient mice (*Ccr2*<sup>-/-</sup>), as expected, both SPM and intPM were drastically reduced in *Ccr2*<sup>-/-</sup> mice bearing ID8 tumors, whereas CCR2 deficiency had little impact on LPM numbers (Fig.S1).

To confirm the continuous contribution of blood monocytes to SPM and intPM populations throughout tumor growth, we used a fluorescent fate-mapping approach. The chemokine receptor Cx3cr1 is expressed by blood monocytes (Geissmann et al., 2003) and previous studies have demonstrated the fate-mapping of monocyte-derived cells using knock-in mice that express a tamoxifen-inducible Cre-recombinase from the Cx3cr1 locus (*Cx3cr1*<sup>CreER</sup>), crossed to mice expressing a ubiquitous lox-STOP-lox reporter cassette (Yona et al., 2013). As expected, we did not detect Cx3cr1 expression in steady-state LPM using the *Cx3cr1*<sup>egfp/+</sup> reporter mice, however, high levels of Cx3cr1 expression were observed in SPM and intermediated levels in intPM (Fig.S1), reflecting the likely monocyte origins of these cells. We crossed *Cx3cr1*<sup>CreER</sup> mice with Rosa26-lsl-tdRFP reporter mice (*Cx3cr1*<sup>CreER</sup>:R26-tdRFP) and injected these mice with ID8 cells to track monocyte-derived cells during tumor growth. Six weeks after injection of ID8 cells, mice were given a single dose of 4-OHT by oral gavage (p.o.) and RFP expression in TAM subsets was measured by flow cytometry ten days later. These experiments showed strong RFP labelling in SPM and intPM within 10 days of 4-OHT administration, with very little labelling of LPM (Fig.1F,G). These data clearly demonstrated the contribution of blood monocytes to SPM and intPM during tumor growth, even within this short time frame.

### ***Transcriptional profiling of TAM.***

To evaluate the impact of the tumor-microenvironment on PM phenotype, we performed global gene expression analysis using microarrays on bulk PM from naïve mice and at different time points during tumor progression. We isolated naïve F4/80<sup>hi</sup> PM and TAM at 5, 12 and 21 days during ID8 tumor development by flow cytometry (Fig.S2). Total RNA was extracted and samples were analyzed using MoGene 1.0st microarrays. RMA normalised data were filtered and analyzed for variations in gene expression. The heatmap in figure S2B shows the 1000

most variable genes in the dataset. To extract differentially expressed genes (DEGs) between naïve PM and TAM at the different time points, we used Anova with an adjusted p value and a threshold of 1.5 fold change (FC). We then used Gene Ontology (GO) enrichment analysis to identify pathways affected in TAM at different time points (Fig.1H,I). DEGs are represented by edges (green = up; blue = down) and individual GO terms are represented by nodes. GO terms that are similar, as indicated by the intersection of DEGs in a given GO term, are closer to each other. This generates clusters of similar GO terms indicating common biological processes within the cluster. This analysis revealed a major cluster of upregulated genes related to immunity in TAM after 5 days (Fig.1H), possibly reflecting a tumoricidal response triggered by resident PM in response to ID8 cells. However, after 21 days, when tumors had become more established, the gene expression profile of TAM more closely resembled the phenotype of naïve PM (Fig.S2). At this later time point, there was an upregulation of distinct gene clusters, including a large cluster of genes related to the innate immune response and tumor necrosis factor (TNF) signaling, in keeping with previous data showing an important role for TNF in this model (Charles et al., 2009; Hagemann et al., 2006), and also a distinctive cluster of genes associated with cholesterol metabolism and efflux (Fig.1I). To confirm the enrichment of genes related to cholesterol homeostasis, we merged several published genesets (Rayner et al., 2011) and known hallmarks to generate an extended gene list representing cholesterol homeostasis. This compiled geneset also showed a significant enrichment in TAM and among the up-regulated genes were known actors in cholesterol metabolism and efflux, including; *Abcg1*, *Ldlr*, *Pparg*, *Hmgcs1*, *Hmgcr*, *Srebf2* (Fig.S2).

### ***Increased membrane cholesterol efflux in TAM.***

Changes in membrane cholesterol content have been shown to dramatically affect macrophage activation in response to pro-inflammatory stimuli, such as bacterial lipopolysaccharide (LPS) (Fessler and Parks, 2011). This is thought, at least in part, to be due to the depletion of cholesterol rich membrane micro-domains, also called lipid rafts, which act as signaling platforms for certain receptors. But membrane cholesterol influences multiple facets of membrane structure and dynamics that can also affect receptor signaling. To confirm the finding that cholesterol efflux pathways were upregulated in TAM, we sought to measure effects on cholesterol membrane content in TAM from ID8 tumor bearing mice. Cholesterol rich membrane micro-domains are commonly measured using cholera toxin B (CTB) staining, which binds to ganglioside GM1, the accumulation of which is linked with membrane cholesterol content. We isolated naïve PM and TAM at 5 and 21 days after injection of ID8 cells, stained the cells with Alexa Fluor 488-conjugated CTB and analyzed them by confocal microscopy. We observed that CTB staining was similar in naïve PM and TAM isolated at 5 days, but was significantly decreased in TAM after 21 days of tumor growth (Fig.2A,B),

indicating that the tumor-microenvironment may promote the depletion of cholesterol rich membrane micro-domains in TAM, in accordance with the upregulation of genes regulating cholesterol efflux in these cells (Fig.1I). To test if ID8 tumor cells had a direct effect on macrophage cholesterol efflux, we co-cultured ID8 cells with bone marrow-derived macrophages (BMDM) *in vitro*. After just one hour of co-culture, there was a significant decrease of CTB staining in BMDM (Fig.2C,D), indicating tumor cells actively promoted the depletion of membrane cholesterol in macrophages. To test if factors secreted by tumor cells were responsible for this effect, we incubated BMDM with conditioned medium obtained from ID8 cell cultures (ID8-CM). This also resulted in a rapid reduction in CTB staining, that was almost equivalent to the effects of methyl- $\beta$ -cyclodextrin (MCD), which extracts cholesterol from cell membranes (Ostrom and Liu, 2007) (Fig.2E,F). Although CTB is commonly used to measure cholesterol rich membrane micro-domains, this is a rather indirect measure of membrane cholesterol. Another method to assess membrane cholesterol content exploits the highly ordered structure of cholesterol-rich membrane microdomains by the use of phase-sensitive fluorescent probes such as Laurdan and di-4-ANEPPDHQ (Owen et al., 2011; Sonnino and Prinetti, 2013). These molecules adapt their emission wavelength based on local membrane order, which is a direct reflection of cholesterol content, independently of membrane-associated proteins. To confirm our findings, we labelled macrophages with di-4-ANEPPDHQ and found a significant decrease of membrane order in the presence of ID8-CM (Fig.2G,H). These assays confirmed that the reduction in CTB staining observed after ID8-CM treatment, correlated with alterations in membrane order that reflect reduced levels of membrane cholesterol. Furthermore, we measured total cholesterol levels in macrophages cultured in the presence or absence of tumor cell-conditioned medium and observed a significant decrease of total cellular cholesterol (Fig.2I). Finally, to directly measure cholesterol efflux from macrophages, we loaded BMDM with thymidine ( $^3\text{H}$ )-labelled cholesterol and measured its efflux into the culture media after addition of the apolipoprotein A1 (ApoA1). Membrane cholesterol efflux is mediated by the transfer of cholesterol to lipoproteins through ABC transporters (Zhao et al., 2010), in the case of ApoA1 this occurs through the transporter ABCA1. These assays showed that addition of ID8-CM significantly increased cholesterol efflux from macrophages, which was reversed in BMDM from ABCA1-deficient mice (*Abca1*<sup>-/-</sup>), demonstrating that this was due to an increased efflux of membrane cholesterol (Fig.2J).

### ***Tumor cell-derived hyaluronic acid (HA) drives cholesterol efflux in macrophages.***

The studies described above showed that ID8 cells increased cholesterol efflux from macrophages. This effect could be recapitulated with conditioned medium but not with fixed cells (Fig.3A), indicating that cholesterol efflux is promoted by a secreted factor. In order to further characterize this factor, we exposed ID8-CM to a series of treatments, including ultra-

centrifugation, boiling (95°C for 5 min), repeated freeze/thaw cycles, DNase and proteinase K, none of which had any impact on the ability of ID8-CM to deplete CTB staining in macrophages (data not shown). However, size fractionation of ID8-CM with cut-offs at 3, 10, 30 or 100 kDa, revealed that this activity was present in a fraction with a molecular weight above 100 kDa (Fig.3B). Several previous studies have shown that the extracellular matrix (ECM) component hyaluronic acid (HA) can be produced by tumor cells and has been linked with increased tumor progression (Chanmee et al., 2016). HA also forms high molecular weight oligomers (>100 kDa) with distinct biological activity (Gomez-Aristizabal et al., 2016; Kolapalli et al., 2016; Rayahin et al., 2015). Furthermore, receptors for HA are expressed by TAM, namely CD44 and Lyve-1 (Chanmee et al., 2016; Turley et al., 2002). To test the hypothesis that HA in ID8-CM contributed to the effects on membrane cholesterol content, we treated ID8-CM with hyaluronidase (HAse) to degrade HA. Indeed, ID8-CM treated with HAse was no longer able to deplete CTB staining in macrophages (Fig.3C). Conversely, when HA of different molecular weights was added to macrophages in normal culture medium, we observed a reduction of CTB staining with increasing molecular weight (Fig.3D). These experiments suggested that high molecular weight HA produced by ID8 cells promotes membrane cholesterol depletion in macrophages. Given that HA is an important component of ECM in many cancers, including EOC (Kolapalli et al., 2016), this suggests HA could affect the phenotype of TAM through membrane cholesterol depletion.

#### ***Cholesterol efflux promotes IL-4 mediated macrophage reprogramming.***

Depletion of membrane cholesterol has been shown to profoundly affect macrophage activation in response to pro-inflammatory stimuli (Fessler and Parks, 2011; Pradel et al., 2009), which suggests that cholesterol efflux in TAM could affect their programming by signals in the tumor-microenvironment. In some instances, TAM have been shown to exhibit a tumor-promoting phenotype that can be driven by Th2 cytokines such as IL-4 or IL-13, and are skewed away from the pro-inflammatory and immunostimulatory activation state, for example induced by Th1 cytokines such as IFN $\gamma$  (DeNardo et al., 2009; Murray et al., 2014). To test the effects of ID8 cells on macrophage reprogramming, we stimulated BMDM with IL-4 or IFN $\gamma$  in the presence or absence of ID8-CM and measured the induction of IL-4 and IFN $\gamma$  gene expression, respectively. ID8-CM pre-treatment profoundly increased the expression of IL-4 induced genes; *Arg1*, *Retnla*, *Chi3l3* and *Mrc1* (Fig.4A). In contrast, ID8-CM inhibited the IFN $\gamma$  induced expression of *Nos2* and *Il12b* (Fig.4B), as well as other IFN $\gamma$ -regulated genes including *Cxcl9*, *Cxcl10* and *Ciita* (Fig.S3), demonstrating that ID8-CM promoted macrophage programming towards an IL-4 induced pro-tumor phenotype. Similar results were obtained after co-culture of ID8 cells with BMDM or with IL-13 treatment, which also signals through the

IL4 receptor alpha chain (IL4RA) (Fig.S3). These effects were restricted to the high molecular weight (>100 kDa) fraction of ID8-CM (Fig.S3) and were not sensitive to freeze/thaw cycles, boiling, ultracentrifugation, DNase or proteinase K treatment (data not shown).

To evaluate the effects of membrane cholesterol depletion on macrophage reprogramming we used several mechanistically distinct treatments to induce cholesterol efflux, in comparison with ID8-CM; 9-cis-retenoic acid (9cRA) upregulates expression of ABC transporters and thereby induces cholesterol efflux (Ricote et al., 2004), whereas high-density lipoprotein (HDL) and ApoA1 strip cholesterol directly from the cell membrane (Zhao et al., 2010). Treatment of BMDM with 9cRA, HDL or ApoA1 resulted in similar levels of reduction in CTB staining as seen upon ID8-CM treatment (Fig.4C), as well as increased IL-4 induced gene expression, while inhibiting IFN $\gamma$  induced genes (Fig.4D). In contrast, the addition of exogenous cholesterol to BMDM, reduced the effects of ID8-CM on IL-4 induced *Arg1* and IFN $\gamma$  induced *Nos2* expression (Fig.S3).

These data suggested that membrane cholesterol depletion promotes IL-4 mediated macrophage activation and abrogates IFN $\gamma$  signalling. To directly test the role of cholesterol efflux in macrophage reprogramming by ID8-CM, we used BMDM from mice with a combined myeloid deficiency in the ABCA1 and ABCG1 reverse cholesterol efflux transporters (*Abca1/g1<sup>ΔLyz2</sup>*). Treatment with ID8-CM or other membrane cholesterol-depleting agents failed to reduce CTB staining in BMDM from *Abca1/g1<sup>ΔLyz2</sup>* mice (Fig.4E), which indeed reversed the increase in IL-4 induced gene expression by ID8-CM and the inhibition of IFN $\gamma$  induced genes (Fig.4F), indicating that membrane cholesterol efflux through ABCA1 and/or ABCG1 promoted IL-4 mediated macrophage activation in the presence of ID8-CM.

### ***Tumor-induced macrophage reprogramming is STAT6 and PI3K dependent.***

To further characterize the mechanisms behind increased IL-4 induced gene expression in the presence of ID8-CM and how this may relate to cholesterol efflux, we analyzed IL-4 receptor signaling pathways. First, we observed no increase in the expression levels of the IL-4 receptor (IL4RA) on macrophages treated with ID8-CM (data not shown), but immunofluorescent staining illustrated an increased intracellular clustering of the receptor that suggested endosomal accumulation, which previously has been shown to promote downstream signaling (Gandhi et al.; Kurgonaite et al.) (Fig S4). We therefore measured activation of signaling pathways downstream of the IL-4 receptor. IL-4 induced gene expression is regulated by JAK-mediated phosphorylation of the STAT6 transcription factor. Treatment of BMDM with ID8-CM increased levels of activated STAT6 (pY-STAT6) in response to IL-4 (Fig.5A,D; Fig.S4) while reducing the accumulation of phosphorylated STAT1 (pY-STAT1), upon IFN $\gamma$  activation (Fig.5B,E; Fig.S4). As expected, IL-4 induced gene expression in the presence of ID8-CM was

abolished in BMDM derived from STAT6 deficient mice (*Stat6*<sup>-/-</sup>) (Fig.S4). IL-4 signaling also activates PI3K, which was recently shown to be an important pathway for the tumor-promoting functions of TAM (Kaneda et al., 2016a; Kaneda et al., 2016b), furthermore, increased PI3K signaling has been shown to promote IL-4 induced gene expression in macrophages (Rauh et al., 2005). To assess PI3K activation we measured phosphorylation of the downstream kinase Akt/PKB. We observed a marked increase in serine 473 phosphorylation of Akt (pS-Akt) in the presence of ID8-CM (Fig.5C,F; Fig.S4), this correlated with increased accumulation of phosphatidylinositol (3,4,5)-triphosphate (PIP<sub>3</sub>), the product of PI3K activity, as measured by confocal microscopy (Fig.S4). To determine the contribution of PI3K to IL-4 mediated reprogramming in the presence of ID8-CM, we treated cells with the PI3K inhibitor LY294002. As expected, LY294002 treatment blocked the increase in pS-Akt by ID8-CM (Fig.5G) and also abrogated the increase in IL-4 induced *Arg1* and *Chi3l3* expression (Fig.5H,I; Fig.S4), indicating that PI3K activity was critical for ID8-CM induced reprogramming. To determine the role of cholesterol efflux in STAT6 and PI3K activation, we again treated macrophages with 9cRA and ApoA1 to deplete membrane cholesterol, both treatments resulted in similar increases in the accumulation of pY-STAT6 and pS-Akt (Fig.5J,K). Furthermore, macrophages lacking the ABCA1 and ABCG1 cholesterol efflux transporters failed to increase pY-STAT6 and pS-Akt upon treatment with ID8-CM (Fig.5L,M). In addition, increased pY-STAT6 and pS-Akt accumulation was restricted to the high molecular weight (>100 kDa) fraction of ID8-CM and could be reversed by Hase treatment (Fig.S4), indicating that HA-mediated cholesterol efflux promoted increased STAT6 and Akt activation.

The specific accumulation of pS-Akt in the presence of ID8-CM was intriguing, serine 473 phosphorylation of Akt is mediated by mammalian target for rapamycin complex 2 (mTORC2) (Jacinto et al., 2006), which is activated by PI3K through PIP<sub>3</sub> accumulation (Liu et al., 2015). Interestingly, mTORC2 was also recently shown to promote IL-4 induced macrophage activation in response to metabolic stress (Huang et al., 2016). Thus, we hypothesised that ID8-CM induced PIP<sub>3</sub> accumulation could activate mTORC2-mediated pS-AKT phosphorylation and increase IL-4 induced gene expression. In the absence of any specific mTORC2 inhibitors, to test the role of the mTORC complex we used rapamycin, which blocks mTORC1, and Torin which blocks both mTORC1 and mTORC2. Rapamycin treatment only partially inhibited ID8-CM induced pS-Akt accumulation in macrophages, however, Torin treatment completely inhibited ID8-CM induced pS-Akt phosphorylation in a dose-dependent manner (Fig.5N,O), suggesting that mTORC2 activity is required for ID8-CM induced pS-Akt accumulation in macrophages.

In summary, IL-4 induced macrophage activation or reprogramming in response to ID8-CM requires PI3K-mTORC2-Akt activity and is driven by STAT6.



### ***IL-4 induced STAT6 and PI3K signaling in TAM drives tumor progression in EOC.***

To test the relevance of these pathways for TAM and tumor progression *in vivo*, we revisited our characterisation of TAM in ID8 tumors. Our conclusions from the data presented in figure 1, was that monocyte-derived TAM gradually replaced resident macrophages during tumor progression and that TAM showed an enrichment for cholesterol efflux pathways (Fig.1I). However, these analyzes were performed on bulk TAM populations, including resident PM and monocyte-derived TAM. To refine our analysis and determine the specific gene expression signature of monocyte-derived cells, we isolated F4/80<sup>lo</sup> CCR2<sup>+</sup> monocytes (MN), alongside F4/80<sup>hi</sup> LPM, which were further divided into Tim4<sup>+</sup> and Tim4<sup>-</sup> subsets (Fig.S5). Tim4 was previously shown to be a marker for proliferative, self-renewing LPM (Rosas et al., 2014), whereas Tim4<sup>-</sup> F4/80<sup>hi</sup> cells represent monocyte-derived LPM, which are CCR2-dependent (Fig.1; Fig.S5). First, we collected these 3 populations from naïve mice and at different time points during tumor progression for microarray analysis. We performed a pairwise comparison between the 3 populations in naïve mice and extracted a specific gene signature for each subset, applying a 1.5 FC threshold and a p-value of 0.05. Using the Minimal method (pairwise[Mean(test)/Mean(ref)]), we identified sets of 553 genes specific for MN, 131 for Tim4<sup>+</sup> PM and 84 for Tim4<sup>-</sup> PM (Table S1). Given that CCR2 was the highest DEG between Tim4<sup>-</sup> and Tim4<sup>+</sup> populations, this strongly supported the monocytic origin of Tim4<sup>-</sup> cells, in keeping with our previous analysis (Fig.S5). We then used these gene sets to perform enrichment analysis (GSEA) with DEGs from the equivalent 3 subsets in ID8 tumor-bearing mice. This analysis showed a significant down-regulation of the naïve Tim4<sup>+</sup> PM gene signature and a strong enrichment of the MN and Tim4<sup>-</sup> gene signatures in Tim4<sup>+</sup> TAM (Fig.6A), supporting our conclusion that the tumor-microenvironment promotes the replacement of resident PM with MN-derived cells that acquire a resident-like phenotype, including expression of Tim4 (Fig.1; Fig.S5). To determine the specific genes associated with this phenotype, we extracted the leading edges (LEs) for this enrichment, that is the genes most strongly associated with the enrichment of the MN gene signature in Tim4<sup>+</sup> TAM. We identified 173 LEs that were enriched at all time points in Tim4<sup>+</sup> TAM (Fig.6B; Table S2), which we then used for Ingenuity Pathway Analysis (IPA). The most significant pathway associated with these genes was the IL-4 pathway (Fig.6B), suggesting that IL-4 in the tumor-microenvironment could be an important upstream regulator for the development of the monocyte-derived TAM phenotype.

To confirm the role of IL-4 signaling in tumor progression *in vivo*, we treated ID8 tumor-bearing mice with an IL-4 receptor blocking monoclonal antibody ( $\alpha$ IL4ra) and monitored tumor progression. Treatment with  $\alpha$ IL4ra significantly reduced ID8 tumor growth *in vivo* (Fig.6C), suggesting that IL-4 signaling is an important factor for tumor progression in this model. Furthermore, chimeric mice with hematopoietic deficiency in STAT6 (*Stat6*<sup>-/-</sup>) or PI3K (*Pik3cd*

<sup>-/-</sup>), also showed significantly reduced tumor growth (Fig.6D,E), indicating that both signaling pathways in tumor stromal cells are important factors for tumor progression. To evaluate the impact of these pathways on TAM phenotype *in vivo*, we sorted bulk TAM from *Pik3cd<sup>-/-</sup>* chimeric mice by flow cytometry and isolated RNA for microarray analysis. Expression of *Arg1*, *Il10*, *Ccl2* and *Stab1*, which have previously been shown to be upregulated in TAM, were significantly downregulated in macrophages from *Pik3cd<sup>-/-</sup>* chimeric mice compared to controls (Fig.S5), suggesting that PI3K activation contributes to the TAM phenotype. To further analyze the impact of PI3K activation on TAM, we generated a gene set from all DEGs between naïve PM and TAM from wild-type mice. Subsequent GSEA showed a significant enrichment for genes expressed by naïve PM in *Pik3cd<sup>-/-</sup>* cells (Fig.6F), confirming that PI3K activity contributes to the promotion of the TAM phenotype. In addition, there was no enrichment of genes associated with the IL-4 dependent TAM phenotype (Fig.6G), described above (Fig.6B; Table S2). These data indicated that PI3K is an important regulator of this gene set in TAM. Interestingly, using an established gene set for tumoricidal phenotype (GSE26912), which was enriched in naïve PM compared to TAM, we also observed an enrichment in *Pik3cd<sup>-/-</sup>* TAM compared to wild-type cells, suggesting that these cells retained a more tumoricidal phenotype in the absence of PI3K activation (Fig.S5). Finally, to evaluate the role of cholesterol efflux in TAM *in vivo*, we established ID8 tumors in mice with a myeloid-specific deletion of both ABCA1 and ABCG1 (*Abca1/g1<sup>ΔLy22</sup>*). ID8 tumor progression was significantly impaired in *Abca1/g1<sup>ΔLy22</sup>* mice compared to littermate controls (Fig.6H). Furthermore, microarray analysis of TAM sorted from these mice showed a significant downregulation of genes associated with the IL-4 dependent TAM phenotype and a positive enrichment for tumoricidal genes (Fig.6I; Fig.S5), reflecting the phenotype of PI3K deficient TAM.

Collectively, these data showed that IL-4 signaling in TAM plays an important role in tumor progression in this model. Furthermore, the PI3K pathway and increased cholesterol efflux, contribute significantly to the functional polarization of TAM and tumor progression *in vivo*.

## Discussion

It is now well appreciated that tumor-associated macrophages (TAM) can play an important role in cancer progression. TAM contribute to tumor progression by various mechanisms, including immune-suppression and trophic functions, supporting angiogenesis, cell proliferation, invasion and metastasis. For example, increased expression of arginase I (Arg1) in TAM, depletes arginine which is required by activated T cells and consequently increases polyamine synthesis, which supports cancer cell proliferation. However, macrophages also possess intrinsic anti-tumor potential, through direct tumoricidal functions and orchestrating

anti-tumor immunity, which may be particularly relevant in response to therapy (Bonnotte et al., 2001; Hagemann et al., 2008; Mytar et al., 1999). But the mechanisms by which TAM become polarized towards pro-tumor functions remain poorly understood.

Here we have studied TAM in a mouse model of epithelial ovarian cancer (EOC), that reflects the peritoneal spread of high-grade serous ovarian cancer (HGSC). In mice, the peritoneal cavity contains a major resident macrophage population of embryonic origin (large peritoneal macrophages; LPM), as well as a minor population of monocyte (MN)-derived macrophages (small peritoneal macrophages; SPM). During tumor progression we showed that MN-derived TAM accumulate and gradually replace resident macrophages in the peritoneal cavity. We then analyzed the global changes in gene expression in TAM over time using microarrays and used pathway analysis to reveal changes in gene expression linked with different pathways and biological functions. At early time points, TAM displayed a more pro-inflammatory gene signature, which strongly distinguished them from naïve resident PM. However, in established tumors, TAM acquired a phenotype more closely resembling resident PM, which suggested a dynamic reprogramming of TAM phenotype during tumor progression.

Among the pathways upregulated in TAM from established tumors compared to naïve PM was a cluster of genes related to cholesterol metabolism and reverse cholesterol efflux. Reverse cholesterol efflux in macrophages is regulated by membrane cholesterol efflux transporters, such as ABCA1 and ABCG1. These transporters regulate the levels of cholesterol in the plasma membrane, which has a profound influence on macrophage responses to extracellular stimuli. For example, ABCA1 deficient macrophages accumulate cholesterol in the membrane and are hyperresponsive to pro-inflammatory stimuli, such as bacterial lipopolysaccharide (LPS) (Fessler and Parks, 2011; Pradel et al., 2009). This is thought to be due to the increase in cholesterol-rich membrane microdomains, also called lipid rafts, which are required to promote TLR4-signaling. However, previous studies have also shown that ABCA1-deficient macrophages are hyporesponsive to other stimuli, including IL-4 and IL-13 (Pradel et al., 2009). Interestingly, ABCG1 deficiency in macrophages was shown to increase their pro-inflammatory phenotype and reduce growth of subcutaneous tumors in mice fed on a high-fat diet (Sag et al., 2015), suggesting that cholesterol accumulation in tumor-associated macrophages can abrogate their pro-tumor functions.

Here, we showed that ovarian cancer cells actively promoted membrane cholesterol efflux in macrophages, which was associated with increased IL-4 signaling and inhibition of IFN $\gamma$ -induced gene expression, resulting in transcriptional and functional reprogramming of TAM. Depletion of membrane cholesterol in macrophages increased PI3K activity and mTORC2-mediated Akt phosphorylation. Both PI3K and mTORC2 have previously been linked with IL-4 mediated macrophage activation in different contexts (Huang et al., 2016; Rauh et al., 2005). Furthermore, PI3K was recently shown to be a critical pathway to maintain the pro-tumor

functions of TAM (Kaneda et al., 2016a; Kaneda et al., 2016b). The exact mechanism by which membrane cholesterol regulates PI3K/mTORC2 activation remains to be elucidated. Perhaps cholesterol-rich membrane microdomains are required to recruit negative regulators of PI3K activity, such as the lipid phosphatase SHIP-1. Previous studies have suggested that SHIP may reside in detergent-resistant membrane fractions (Galandrini et al., 2002) and SHIP-1 is known to inhibit IL-4 signaling in macrophages (Rauh et al., 2005).

The distinct metabolic environment of tumors has long been suggested to influence the phenotype of tumor-infiltrating immune cells, rendering them hyporesponsive and contributing to immune-suppression. Cancer cells rely heavily on cholesterol, which they can scavenge from the tumor-microenvironment through upregulation of apolipoproteins and their receptors (Guillaumond et al., 2015; Podzielinski et al., 2013; Villa et al., 2016). This may lead to cholesterol depletion in tumor-stromal cells, and particularly TAM which express high levels of the ABCA1 and ABCG1 efflux transporters. Our *in vitro* studies suggest hyaluronic acid (HA) could be an important factor produced by cancer cells that promotes this process. HA is a major component of the extracellular matrix (ECM) in many human cancers, including ovarian cancer and in many cases the degree of HA accumulation strongly correlates with poor prognosis (Kolapalli et al., 2016; Sironen et al., 2011). Macrophages express at least two distinct receptors for HA; CD44 and Lyve1. Interestingly, CD44 signaling has previously been associated with PI3K activation in TAM (Lenart et al., 2017). Co-incidentally, PI3K also upregulates ABCA1 expression in macrophages (Chen et al., 2012; Okoro et al., 2016), potentially creating a feed-forward loop for enhanced cholesterol efflux and IL-4 mediated reprogramming.

In summary, we describe an important role for membrane cholesterol efflux in the regulation of macrophage activation state in the tumor-microenvironment. Depletion of membrane cholesterol renders macrophages hyperresponsive to pro-tumor signals, such as IL-4, but refractory to activation by the anti-tumor cytokine IFN $\gamma$ . We propose that cholesterol efflux pathways may represent novel targets to abrogate the pro-tumor functions of TAM while retaining potentially beneficial anti-tumor effects in response to therapy.

### **Limitations of this study**

Despite functional data demonstrating the contribution of IL-4 signaling to tumor progression in this model, IL-4 was not detectable in ascites from tumor-bearing mice, indicating this cytokine is produced at very low levels and consumed rapidly. This illustrates the significance of enhanced sensitivity of TAM to IL-4, which is increased by several orders of magnitude due to membrane cholesterol depletion. These studies are likely to be relevant to human ovarian

cancer, where IL-4 expression has been associated with poor clinical outcome (Candido et al.; Clendenen et al.; Munster et al., 1998). The source of IL-4 in this context remains unknown. While other cancer cells, including prostate, breast and bladder, have been shown to express IL-4 (Conticello et al., 2004), conditioned medium from ID8 cells alone was not sufficient to activate IL-4 signaling in macrophages. Other potential sources of IL-4 include CD4 T cells (DeNardo et al., 2009), eosinophils (Kratovich et al., 2015), innate lymphocytes or macrophages themselves.

The precise mechanism by which the activity of ABC transporters is upregulated in TAM also remains to be elucidated. Cholesterol efflux was rapidly induced upon exposure to tumor-cell conditioned medium and no increase in *Abca1* or *Abcg1* mRNA was observed (data not shown), suggesting a post-transcriptional mechanism. However, cholesterol efflux was dependent PI3K activation associated with increased Akt serine 473 phosphorylation. Other studies have shown serine 473 phosphorylation of Akt regulates the translocation of ABC transporters to the plasma membrane (Huang et al.). This may offer a mechanistic explanation for increased ABC activity in TAM.

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## **Author contributions**

PG, JRV, AE and TL designed the experiments. PG, JRV, AE performed the experiments with help from OR, VG, CV, MB, NAA. Bioinformatics analysis was performed by MM, TPVM, MD, OP, TU and JLS. MVE performed cholesterol efflux assays. MT provided *Pik3cd*<sup>-/-</sup> mice. PG, JRV, AE and TL wrote the manuscript.

## Declaration of Interests

The authors declare no competing interests.

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## Figure Legends

### **Figure 1. Ontogeny and phenotype of TAM during ID8 tumor development.**

**(A)** Analysis of peritoneal macrophages (PM) by flow cytometry after intra-peritoneal (i.p.) injection of  $10^6$  ID8-Luc cells. PM were gated as; live, single cells (FSC-H versus FSC-W), CD45.2<sup>+</sup>, Lin<sup>-</sup> (NK1.1 Ly6G CD5 CD19), CD11b<sup>+</sup>, CD64<sup>+</sup>. LPM were subsequently gated as F4/80<sup>hi</sup> MHCII<sup>-</sup> (blue), intPM as F4/80<sup>int</sup> MHCII<sup>int</sup> (orange) and SPM as F4/80<sup>-</sup> MHCII<sup>hi</sup> (green).

**(B)** Total numbers of LPM, intPM and SPM during tumor growth. **(C)** Shielded chimeras reconstituted with mixed bone marrow to analyze ontogeny of PM after engraftment with ID8-Luc cells. Host mice (CD45.1) were placed in protective lead shield with only the hind legs exposed, before irradiation with 9 Gy. The following day, mice were reconstituted with a mixture of bone marrow cells from *Ccr2*<sup>-/-</sup> (CD45.2) and CD45.1/2 congenic mice, at a ratio of 4:1. 5 weeks after reconstitution, mice were injected with ID8-Luc cells. A further 8 weeks after tumor inoculation, PM were collected for analysis. **(D)** Analysis of PM from chimeric mice by flow cytometry; CD45.1 and CD45.2 expression was analyzed on LPM (blue), intPM (orange) and SPM (green), as described above. **(E)** Proportion of chimerism was calculated relative to blood monocytes. **(F,G)** Fate mapping of Cx3cr1-expressing monocytes in ID8 tumor-bearing mice; Cx3cr1<sup>CreER</sup>:R26-tdRFP mice were inoculated with ID8-Luc cells, after 8 weeks 1 mg tamoxifen was administered by oral gavage and RFP expression was analyzed in PM subsets 10 days later. RFP expression within LPM, intPM and SPM is shown. **(H,I)** PM were collected from naïve mice and at 5 or 21 days after tumor inoculation by flow cytometry. Total cellular mRNA was extracted and global gene expression profiles analyzed using microarrays. Gene ontology enrichment was performed on the differentially expressed genes (DEGs) between

naïve PM and TAM at (H) 5 and (I) 21 days, respectively. Graphs are represented as mean  $\pm$  SEM. See also Figures S1 and S2.

**Figure 2. Increased membrane cholesterol efflux in TAM.**

(A,B) Peritoneal macrophages were isolated from naïve mice (Ctrl) or at 5 and 21 days after inoculation with ID8 cells. Cells were seeded in chambered plastic slides and stained with Cholera Toxin B (CTB; green) and TO-PRO-3 (blue). (A) Representative images for all three groups are shown, (B) quantification of corrected total cell fluorescence (CTCF). (C,D) BMDM were co-cultured with ID8 cells for the indicated time points prior to CTB staining, (C) representative images for BMDM alone (Ctrl) and 3 hours after ID8 co-culture and (D) quantification of CTCF. (E-H) BMDM were incubated with ID8-CM overnight, or Methyl- $\beta$ -cyclodextrin for 30 min, prior to CTB staining or di-4-ANEPPDHQ staining; (E) representative images for CTB staining in each condition and (F) quantification of CTCF. (G) Representative images of di-4-ANEPPDHQ staining and (H) quantification of relative changes in GP values (Owen et al., 2011). (I) Total cell cholesterol content was measured in BMDM with and without ID8-CM treatment, using the Amplex Red Cholesterol Assay kit. (J) BMDM from wild-type (WT) or *Abca1*<sup>-/-</sup> mice were loaded with <sup>3</sup>H-labeled cholesterol and subsequently treated or not with ID8-CM in the presence of ApoA1, as an acceptor for cholesterol. <sup>3</sup>H-Cholesterol was measured in the medium as a readout for cholesterol efflux. Graphs are represented as mean  $\pm$  SEM.

**Figure 3. Tumor cell-derived hyaluronic acid (HA) oligomers deplete lipid rafts in macrophages.**

(A) BMDM were co-cultured with live or paraformaldehyde-treated (fixed) ID8 cells, prior to CTB staining, quantification of CTCF is shown. (B) High and low molecular weight fractions of ID8-CM were prepared using Centricon filters with 100 kDa pores, each fraction was compared with unfractionated ID8-CM for effects on CTB staining. (C) BMDM were treated with ID8-CM with and without hyaluronidase (HAse) treatment prior to CTB staining and quantification. (D) BMDM were incubated with HA oligomers of increasing molecular weights before CTB staining and quantification, ID8-CM was used as a positive control. Graphs are represented as mean  $\pm$  SEM.

**Figure 4. Cholesterol efflux promotes IL-4 mediated macrophage reprogramming.**

(A,B) BMDM were treated with or without ID8-CM before stimulation with increasing concentrations of IL-4 or IFN $\gamma$  for 8 hours. (A) Quantitative PCR (qPCR) analysis of IL-4 induced gene expression; *Arg1*, *Chi3l3*, *Mrc1*, *Retnla* and (B) IFN $\gamma$ -induced expression of *Nos2*

and *Ii12b*. (C) BMDM were treated with different cholesterol depleting agents; 9-cis-retenoic acid (9cRA), high-density lipoprotein (HDL) or apolipoprotein A1 (ApoA1), as well as ID8-CM; lipid raft density was subsequently measured by CTB staining, quantification of CTCF is shown. (D) IL-4 (20 ng/ml) induced *Arg1* and IFN $\gamma$  (20 ng/ml) induced *Nos2* expression in BMDM treated with 9cRA, HDL or ApoA1, compared to ID8-CM. (E) BMDM from *Abca1/g1<sup>ff</sup>* and *Abca1/g1 <sup>$\Delta$ Ly22</sup>* mice were incubated with ID8-CM, 9cRA, HDL or ApoA1 and lipid raft density was measure by CTB staining, quantification of CTCF is shown. (F) IL-4 induced *Arg1* and IFN $\gamma$  induced *Nos2* expression in BMDM from *Abca1/g1<sup>ff</sup>* and *Abca1/g1 <sup>$\Delta$ Ly22</sup>* mice with and without ID8-CM treatment. Graphs are represented as mean  $\pm$  SEM. See also Figure S3.

**Figure 5. Tumor-induced reprogramming is STAT6 and PI3K dependent.**

(A-F) BMDM were treated with or without ID8-CM overnight before stimulation with either IL-4 or IFN $\gamma$  for 20 min. Cells were then fixed and stained for p-STAT6, p-STAT1 or p-Akt and analyzed by confocal microscopy; representative micrographs for (A) p-STAT6, (B) p-STAT1 and (C) p-Akt staining are shown (green), nuclei are counterstained with TO-PRO-3 (blue). (D-F) Quantification of CTCF for (D) p-STAT6, (E) p-STAT1 and (F) p-Akt (NS – non stimulated). (G-I) BMDM were incubated with or without ID8-CM in the presence or absence of the PI3K inhibitor LY294002 (1.25  $\mu$ M); (G) Quantification of p-Akt staining by confocal microscopy and (H-I) qPCR analysis of IL-4-induced *Arg1* and *Chi3l3* expression. (J-K) BMDM were incubated overnight with ID8-CM, 9cRA, HDL or ApoA1 before stimulation with IL-4 for 20 min; quantification of (J) p-STAT6 and (K) p-Akt by confocal microscopy. (L-M) BMDM from *Abca1/g1<sup>ff</sup>* and *Abca1/g1 <sup>$\Delta$ Ly22</sup>* mice were treated with ID8-CM before stimulation with IL-4 and quantification of (L) p-STAT6 and (M) p-Akt by confocal microscopy. (N-O) BMDM treated with ID8-CM in the presence or absence of Rapamycin (Rapa) or Torin at the indicated concentrations; (N) quantification of p-Akt by confocal microscopy and (O) western blot analysis of Ser473 and Thr308 Akt phosphorylation (pAkt(Ser) and pAkt(Thr), respectively) in Rapamycin (R) and Torin (T) treated BMDM with and without ID8-CM treatment. Graphs are represented as mean  $\pm$  SEM. See also Figure S4.

**Figure 6. IL-4 induced STAT6 and PI3K signaling in TAM drives tumor progression in vivo.**

(A) Generation of specific gene signatures for Tim4<sup>+</sup> and Tim4<sup>-</sup> F4/80<sup>hi</sup> PM and F4/80<sup>lo</sup> CCR2<sup>+</sup> monocytes (MN) using GeneSign (BubbleGUM) cell-specific signatures were assessed for enrichment using BubbleMap (Spinelli et al., 2015), based on the GSEA algorithm in pairwise comparisons of Tim4<sup>+</sup> PM from naïve mice or ID8 tumor-bearing mice at different time points.

The bubble area is proportional to the absolute value of the normalised enrichment score (NES). The color intensity indicates the false-discovery rate (FDR), modified for multiple testing. The size and intensity of color increases with the enrichment of the gene signature from the matching cell population; the Tim4<sup>+</sup> gene signature is enriched in naïve cells (red), whereas the Tim4<sup>+</sup> and MN gene signatures are enriched in TAM (blue). **(B)** Leading edges (LEs) of the MN gene signature enrichment at different time points (highlighted by colored boxes) were extracted and plotted in a Venn diagram. Ingenuity Pathway Analysis (IPA) was performed on the 173 common LEs, showing IL4 as the top upstream regulator. **(C)** ID8-Luc cells were injected i.p. and mice treated with either IL4R $\alpha$  blocking antibody (anti-IL4R $\alpha$ ) or an isotype control antibody (Iso-ctrl) after 2 weeks; tumor growth was assessed at 4 weeks by *ex vivo* luciferase assay on peritoneal cells. **(D-E)** Cohorts of chimeric mice receiving either wild-type (WT), **(D)** *Stat6*<sup>-/-</sup> or **(E)** *Pik3cd*<sup>-/-</sup> bone marrow cells were injected with ID8-Luc cells and tumor growth assessed after 6 weeks. **(F,G)** TAM were isolated from *Pik3cd*<sup>-/-</sup> chimeric mice and WT controls after 6 weeks of tumor growth by flow cytometry. Microarray analysis was performed to assess geneset enrichment using GSEA. **(F)** Positive enrichment of the naïve PM gene signature in *Pik3cd*<sup>-/-</sup> TAM compared to WT cells and **(G)** negative enrichment of the IL-4 pathway signature of TAM (from **B**) in *Pik3cd*<sup>-/-</sup> cells. ES plots are shown on the left and the 25 most enriched genes on the right. NES, normalised enrichment score; FDR, false discovery rate. **(H)** *Abca1/g1*<sup>ff</sup> and *Abca1/g1* <sup>$\Delta$ Ly22</sup> mice were injected with ID8-Luc cells and tumor growth assessed after 6 weeks. **(I)** GSEA with TAM isolated from *Abca1/g1*<sup>ff</sup> and *Abca1/g1* <sup>$\Delta$ Ly22</sup> mice showing negative enrichment of the TAM associated IL4 pathway in *Abca1/g1* <sup>$\Delta$ Ly22</sup> mice. Graphs are represented as mean  $\pm$  SEM. See also Figure S5.

## STAR Methods

### Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
anti-Nos2 (M-19)	Santa Cruz	sc-650
anti-Arg1 (N-20)	Santa Cruz	sc-18351
HRP-conjugated anti-Rabbit immunoglobulins	DAKO	P044801-2
HRP-conjugated anti-Mouse immunoglobulins	DAKO	P026002-2
anti- $\beta$ -Actin (AC-74)	Sigma	A5316
anti-Akt (40D4)	Cell Signaling	2920
anti-pSer473-Akt (D9E)	Cell Signaling	4060
anti-pThr308-Akt (D25E6)	Cell Signaling	13038
anti-STAT1	Cell Signaling	9172
anti-pTyr701-STAT1	Cell Signaling	9171

anti-STAT6 (M-20)	Santa Cruz	sc-981
anti-pTyr641-STAT6	Cell Signaling	9361
anti-PIP3	Cell Signaling	
Anti-CD124	Novus Biologicals	NBP1-00884
anti-CD11b (M1/70)	BD Biosciences,	563553
anti-CD44 (IM7)	BD Biosciences, eBioscience, BioLegend, and Life Technologies.	
anti-CD45.1 (A20)	BD Biosciences, eBioscience, BioLegend, and Life Technologies.	553775
anti-NK1.1 (PK136)	BioLegend	108724
anti- Ly6G (1A8)	BD Biosciences	560600
anti- CD5 (53-7.3)	BD Biosciences	563194
anti-CD19 (1D3)	BD Biosciences	565076
anti-CD64 (X54-5/7.1)	BioLegend	139311
anti-Ly6C (AL-21)	BD Biosciences	553104
anti-MHCII (M5/114)	eBioscience	56-5321-82
anti-F4/80 (BM8)	BioLegend	123141
anti-Lyve-1 (ALY7)	eBioscience	53-0443-82
anti-rabbit-Alexa488	Invitrogen	A-10040
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
LY294002	R&D Systems	1130
Rapamycin	Merck	553211
Hyaluronic acid	Lifecore Biomedical	HA15M-1
Hyaluronidase	Sigma	H3506
Torin	Sigma	475991
Methyl- $\beta$ -cyclodextrin	Sigma	C4555
High-density lipoprotein	Sigma	L8039
9-cis-retenoic acid	Sigma	R4643
Cholesterol-methyl- $\beta$ -cyclodextrin	Sigma	C4951
Proteinase inhibitor cocktail	Sigma	P8340
Xylazine	Sigma	23076-35-9
PNPP	Sigma	333338-18-4
$\beta$ -glycerophosphate	Sigma	G9422
DTT	Sigma	43819
Apolipoprotein A1	Sigma	A0722
Recombinant mouse IL4	Peptotech	214-14
Recombinant mouse IFN $\gamma$	Peptotech	315-05
Recombinant mouse M-CSF	Peptotech	315-02
Recombinant mouse IL13	Peptotech	210-13
Vybrant Alexa Fluor 488 Lipid Raft Labelling Kit	ThermoFisher Scientific	V34403
TRIzol	ThermoFisher Scientific	15596018
di-4-ANEPPDHQ	ThermoFisher Scientific	D36802
TO-PRO-3	ThermoFisher Scientific	T3605
SYBR Green PCR Master Mix	ThermoFisher Scientific	4312704
IL4R $\alpha$ neutralizing antibody	BD Biosciences	552508
<b>Critical Commercial Assays</b>		
Superscript IV Reverse Transcriptase	Thermo Fisher Scientific	18090050
Amplex Red Cholesterol Assay kit	ThermoFisher Scientific	A12216
<b>Deposited Data</b>		

GSE126079	This paper	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126079">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126079</a>
GSE126080	This paper	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126080">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126080</a>
GSE126098	This paper	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126098">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126098</a>
<b>Experimental Models: Cell Lines</b>		
Bone marrow derived macrophages	This paper	Hagemann et al., 2008
ID8-Luc ovarian surface epithelial cell line	Prof. Frances Balkwill	Barts Cancer Institute, London, UK
<b>Experimental Models: Organisms/Strains</b>		
Mouse: C57Bl/6	Charles River	JAX 000664
Mouse: Abca1 <sup>tm1Jp</sup> Abcg1 <sup>tm1Tail</sup>	Jackson Laboratories	stock number 021067
Mouse: Lyz2 <sup>tm1(cre)lfo</sup>	Jackson Laboratories	stock number 004781
Mouse: Stat6 <sup>-/-</sup>	Dr. Bernard Malissen	CIML, Marseille, FR
Mouse: Abca1 <sup>-/-</sup>	Prof. Miranda Van Eck	Leiden University, NL
Mouse: Cx3cr1 <sup>+eGFP</sup>	Jackson Laboratories	stock number 005582
Cx3cr1 <sup>cre/ERT2</sup>	Dr. Stephan Jung	Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel.
Mouse: Pik3cd <sup>-/-</sup>	Prof. Martin Turner	Babraham Institute, Cambridge, UK
<b>Oligonucleotides</b>		
Arg1; F-CAGAAGAATGGAAGAGTCAG	This paper	N/A
Arg1; R-CAGATATGCAGGGAGTCACC	This paper	N/A
Chi3l3; F-TCACAGGTCTGGCAATTCTTCTG	This paper	N/A
Chi3l3; R-TTTGTCTTAGGAGGGCTTCCTC	This paper	N/A
Ciita; F-CTCAGCCTTAGGAGGGACTTG	This paper	N/A
Ciita; R-GACCTGGATCGTCTCGTGCAG	This paper	N/A
Cph; F-GGCAAATGCTGGACCAAACAC	This paper	N/A
Cph; R-TTAGAGTTGTCCACAGTCGGAGATG	This paper	N/A
Cxcl9; F-TCCTTTTGGGCATCATCTTC	This paper	N/A
Cxcl9; R-TTCCCCTCTTTTGCTTTT	This paper	N/A
Cxcl10; F-GGGCCATAGGGAAGCTTGAA	This paper	N/A
Cxcl10; R-GGATTCAGACATCTCTGCTCATCA	This paper	N/A
Il12b; F-GGAA GCACGGCAGCAGAATA	This paper	N/A
Il12b; R-AACTTGAGGGAGAAGTAGGAATGG	This paper	N/A

Ccl2; F-GCCAGCTCTC TCTTCCTCCA	This paper	N/A
Ccl2; R-CCCAGAAGCATGACAGGGAC	This paper	N/A
Nos2; F-CCCTCCTGATCTTGTGTT GGA	This paper	N/A
Nos2; R-CCACCCGAGCTCCTGGAAC	This paper	N/A
Retnla; F-GTCCCAGTGCATATGGATGAGACCATAGA	This paper	N/A
Retnla; R-ACCTCTTCACTCGAGGGACAGTTGGCAGC	This paper	N/A
<b>Software and Algorithms</b>		
FIJI software	Schindelin, J., et al. 2012	<a href="https://fiji.sc/">https://fiji.sc/</a>
FlowJo cytometric analytical software	Tree Star	
Gene Set Enrichment Analysis (GSEA)	Broad Institute	Subramanian et al., 2005
Affymetrix Expression Console version 1.1 software	Affymetrix	
Prism software	Graphpad	
BioLayout Express3D		Theocharidis et al., 2009
<b>Other</b>		

## Materials

The following materials were employed throughout the study: LY294002, Torin, Rapamycin (Merck); hyaluronic acid, hyaluronidase, methyl- $\beta$ -cyclodextrin, apolipoprotein A1, high-density lipoprotein, 9-cis-retenoic acid and cholesterol-methyl- $\beta$ -cyclodextrin (Sigma); recombinant mouse IL-4, IFN $\gamma$  and M-CSF (Peprotech); IL4R $\alpha$  neutralizing antibody (BD Biosciences).

## Mice

C57Bl/6 mice were obtained from Charles River. All transgenic mouse strains were backcrossed to a C57Bl/6 background. *Abca1<sup>tm1Jp</sup> Abcg1<sup>tm1Tall</sup> (Abca1/g1<sup>if</sup>)* and *Ly22<sup>tm1(cre)lfo</sup> (Ly22<sup>Cre</sup>)* mice were obtained from Jackson Laboratories. *Stat6<sup>-/-</sup>* mice were kindly donated by Dr. Bernard Malissen (CIML, Marseille, FR), *Abca1<sup>-/-</sup>* mice by Prof. Miranda Van Eck (Leiden University, NL) and *Pik3cd<sup>-/-</sup>* mice by Prof. Martin Turner (Babraham Institute, Cambridge, UK). Generation of shielded chimeras was performed as previously described (Scott et al., 2016); briefly, CD45.1 congenic mice were anaesthetised with Ketamine (150 mg/kg) and Xylazine (10 mg/kg) and placed in 6 mm thick lead cylinders, exposing only the hind legs. With the peritoneal cavity protected, mice were irradiated with 9 Gy and reconstituted with 10<sup>7</sup> bone marrow cells from *Ccr2<sup>-/-</sup>* (CD45.2) and CD45.1/2 mice, at a ratio of 4:1. After 5 weeks, chimerism of blood leukocytes was assessed by flow cytometry. All mice were housed under specific pathogen-free conditions and animal experimentation was conducted in strict accordance with good animal practice as defined by the French animal welfare bodies relative



to European Convention (EEC Directive 86/609) and approved by the Direction Départementale des Services Vétérinaires des Bouches du Rhône.

### ***Cell Culture***

Bone marrow derived macrophages (BMDM) were obtained as previously described (Hagemann et al., 2008); briefly, femurs and tibiae from mice aged 8 to 10 weeks were flushed and cells collected by centrifugation at 450 g for 5 min at 4°C. Cells were resuspended in DMEM supplemented with L-glutamine (2 mM), penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco), 10 % heat-inactivated FBS and 10 ng/ml recombinant mouse M-CSF (PeproTech) and cultured at a density of 10<sup>6</sup> cells/ml in non-tissue culture treated plastic dishes (BD Pharmingen) at 37°C and 5 % CO<sub>2</sub>. After 7 days, adherent cells were collected and resuspended in complete DMEM containing 10 ng/ml M-CSF. The ID8-Luc ovarian surface epithelial cell line was kindly provided by Prof. Frances Balkwill (Barts Cancer Institute, London, UK). To obtain ID8 cell-conditioned medium (ID8-CM); 13.75 x10<sup>6</sup> cells in 25 ml were incubated for 72 hours in a 175 cm<sup>2</sup> flasks in DMEM containing 4 % of FCS. Medium was filtered through a 22 µm filter, aliquoted and stored at -80°C.

### ***Immunofluorescence and lipid raft staining***

BMDM were grown in Lab-Tek chambered slides (ThermoFisher Scientific) and fixed with 4 % PFA, permeabilised (0.1 % Triton-X100) and blocked in 5 % BSA with 10 mM glycine. The following primary antibodies were used for incubation during 90 minutes at 4°C; anti-pSTAT1, anti-pSTAT6, anti-pSer473-Akt, anti-pThr308-Akt, anti-PIP<sub>3</sub> (Cell Signaling) or anti-IL4Rα (Novus biologicals). After washing, anti-rabbit-Alexa488 (Invitrogen) and TO-PRO-3 (ThermoFisher Scientific) were added for 60 minutes. Lipid rafts were stained using the Vybrant Alexa Fluor 488 Lipid Raft Labelling Kit, following manufacturer's instructions. Briefly, BMDM were grown and stimulated in Lab-Tek chambered slides. After washing with serum-free DMEM, they were incubated for 10 minutes with Alexa488-conjugated cholera toxin subunit B (CTB) at 4°C, followed by cross-linking with an anti-CTB antibody for 15 minutes at 4°C. Subsequently, the cells were fixed with 4 % Antigenfix (DiaPath) for 10 minutes on ice and nuclei were stained with TO-PRO-3. The di-4-ANEPPDHQ lipid raft staining protocol was adapted from the one described by Owen et al. (Owen et al., 2011). Briefly, culture medium was replaced with fresh, serum-free DMEM containing 2 µl of di-4-ANEPPDHQ (5 µM). Dishes were shaken to ensure good mixing. After 30 min incubation at 37 °C in a humidified 5 % CO<sub>2</sub> atmosphere, cells were fixed with 4 % PFA. Fluorescence was measured by confocal microscopy (Zeiss LSM780 or Leica SP5X) and analyzed with FIJI software. The corrected

total cell fluorescence (CTCF) was measured for each cell in at least 6 different fields of view per well.

### ***Cholesterol measurement***

Total cell cholesterol content was measured in BMDM using the Amplex Red Cholesterol Assay kit (ThermoFisher Scientific), according to the manufacturer's instructions.

### ***Immunoblotting***

BMDM were lysed on ice in lysis buffer supplemented with proteinase inhibitor cocktail, PNPP,  $\beta$ -glycerophosphate and DTT. Separation by SDS-PAGE was followed by blotting on PVDF membrane. Blots were blocked with 5 % skimmed milk in TBS-0.05 % Tween20. The following primary antibodies were used; anti-Nos2, anti-Arg1 (Santa Cruz), anti- $\beta$ -Actin (Sigma), anti-Akt, anti-pSer473-Akt, anti-pTyr701-STAT1, anti-STAT1, anti-pTyr641-STAT6 (Cell Signaling) and anti-STAT6 (Santa Cruz). Primary antibodies were incubated overnight at 4°C and appropriate HRP-conjugated secondary antibodies (DAKO) for 1 hour at room temperature. Chemoluminescence was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific).

### ***Gene expression analysis***

Total cellular RNA was extracted from BMDM using TRIzol and cDNA was synthesised with cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Gene expression was quantified using sequence specific primers in the presence of SYBR Green PCR Master Mix using an ABI 7900HT thermocycler (Applied Biosystems). Reactions were performed in duplicate or triplicate and Ct-values were normalised to the mean Ct-values of cyclophilin. Relative quantification of gene expression was calculated as  $2^{-\Delta\Delta Ct}$  to controls. Primers used: *Arg1*; F-CAGAAGAATGGAAGAGTCAG, R-CAGATATGCAGGGAGTCACC. *Chi3l3*; F-TCACAGGTCTGGCAATTCTTCTG, R-TTTGTCCTTAGGAGGGCTTCCTC *Cph*; F-GGCAAATGCTGGACCAAACAC, R-TTAGAGTTGTCCACAGTCGGAGATG. *Ii12b*; F-GGAA GCACGGCAGCAGAATA, R-AACTTGAGGGAGAAGTAGGAATGG. *Ccl2*; F-GCCAGCTCTC TCTTCCTCCA, R-CCCAGAAGCATGACAGGGAC. *Nos2*; F-CCCTCCTGATCTTGTGTT GGA, R-CCACCCGAGCTCCTGGAAC. *Retnla*; F-GGTCCCAGTGCATATGGATGAGACCA TAGA, R-CACCTCTTCACTCGAGGGACAGTTGGCAGC. *Cxcl9*; F-TCCTTTTGGGCATCATCTTC, R-TTCCCCCTCTTTTGCTTTTT. *Cxcl10*; F-GGGCCATAGGGAAGCTTGAA, R-GGATTCAGACATCTCTGCTCATCA. *Ciita*; F-CTCAGCCTTAGGAGGGACTTG, R-GACCTGGATCGTCTCGTGACG.

### ***Ovarian cancer model***

One million ID8 cells were injected intraperitoneally in the different mouse strains using a 27G syringe. Mice were euthanised at the indicated times and peritoneal lavages were collected for cytometric analysis, *ex vivo* bioluminescence measurement and/or lipid raft staining. Briefly, 9 ml of ice cold PBS was injected intraperitoneally and after a careful massage to detach all the cells in the cavity, peritoneal fluid was collected through a 23G syringe. Tubes were weighed to determine the recovered lavage volume and the cell density was assessed using a Casy cell counter (Innovatis). Cells were centrifugated and resuspended in 1 ml cold PBS. One million cells from each peritoneal lavage were stained for flow cytometry. 50 µl of the 1 ml cell suspension obtained from peritoneal lavage was used for luciferase activity measurements. Cells were plated in a white 96-well plate and 50 µl luciferin was added to each well, Luminescence [photons/s] was measured for each well using the Mithras Microplate Reader (Berthold Technologies).

### ***Flow Cytometry***

Peritoneal lavage cells underwent a short NH<sub>4</sub>Cl red blood cell lysis and were incubated at 4°C for 10 min with the 2.4.G2 antibody to block Fc receptors. The cells were stained with the indicated antibodies for 30 min at 4°C. Dead cells were gated out using SYTOX Blue dead cell stain (Life Technologies). After cell-surface staining, cells were fixed. Analysis was performed using an LSR-II flow cytometer or sorted using an Aria III cell sorter (both BD Biosciences) and data analysis was conducted with the FlowJo cytometric analytical software (Tree Star). Anti-CD11b (M1/70), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), NK1.1, Ly6G, anti-CD5 (53-7.3), anti-CD19 (1D3), anti-CD64, anti-Ly6C (AL-21), anti-F4/80 and anti-MHCII (M5/114) were purchased from BD Biosciences, eBioscience, BioLegend, and Life Technologies.

### ***Microarray Analysis***

RNA samples were hybridised on Affymetrix Mouse 430 2.0 or MoGene 1.0 st chips. Samples were processed as follows: The biotinylated cRNAs were prepared according to a double amplification protocol using MessageAmp™ II aRNA Amplification Kit (Ambion). The images of the chips were generated with Affymetrix software AGCC version 3.2. The expression data was then extracted with the Affymetrix Expression Console version 1.1 software using the RMA (log2 scale) and MAS5 (linear scale) algorithms. Gene Set Enrichment Analysis (GSEA, Broad Institute) (Subramanian et al., 2005) was used to examine differentially expressed genes (DEGs). The output of GSEA is an enrichment plot (ES), a normalised enrichment score (NES) which accounts for the size of the gene set being tested, a p-value, and an estimated False Discovery rate (FDR). We computed P values using 1,000 permutations for each gene set and

corrected them with the false-discovery rate (FDR) method. When several probe sets were present for a gene, the mean of the probe set was used. Cell-specific gene sets were generated by performing pairwise comparisons between DEGs from different populations, applying a 1.5 FC threshold and a p value of 0.05, using the Minimal (pairwise[Mean(test)/Mean(ref)]) method. Sample correlation analysis was performed based on Pearson's correlation coefficients using BioLayout Express3D (Theocharidis et al., 2009). GO enrichment analysis was applied using the Cytoscape plug-in BiNGO (v2.44) (Maere et al., 2005) with FDR q-value threshold of 0.05 as default. The Cytoscape plugins Enrichment Map (v1.1) (Merico et al., 2010) and Word Cloud (Oesper et al., 2011) were used to visualize the GO networks. Accession numbers for datasets are; GSE126079, GSE126080, GSE126098.

### ***Statistical Analysis***

Graphs were made and statistical analysis was performed using Prism software (Graphpad). All quantitative data are presented as mean  $\pm$  SEM. Statistical significance was calculated using Student's t-test, Wilcoxon-Mann-Whitney test, Chi-square test for contingency tables or One-Way ANOVA. P values <0.05 were considered as significant.

Figure 1

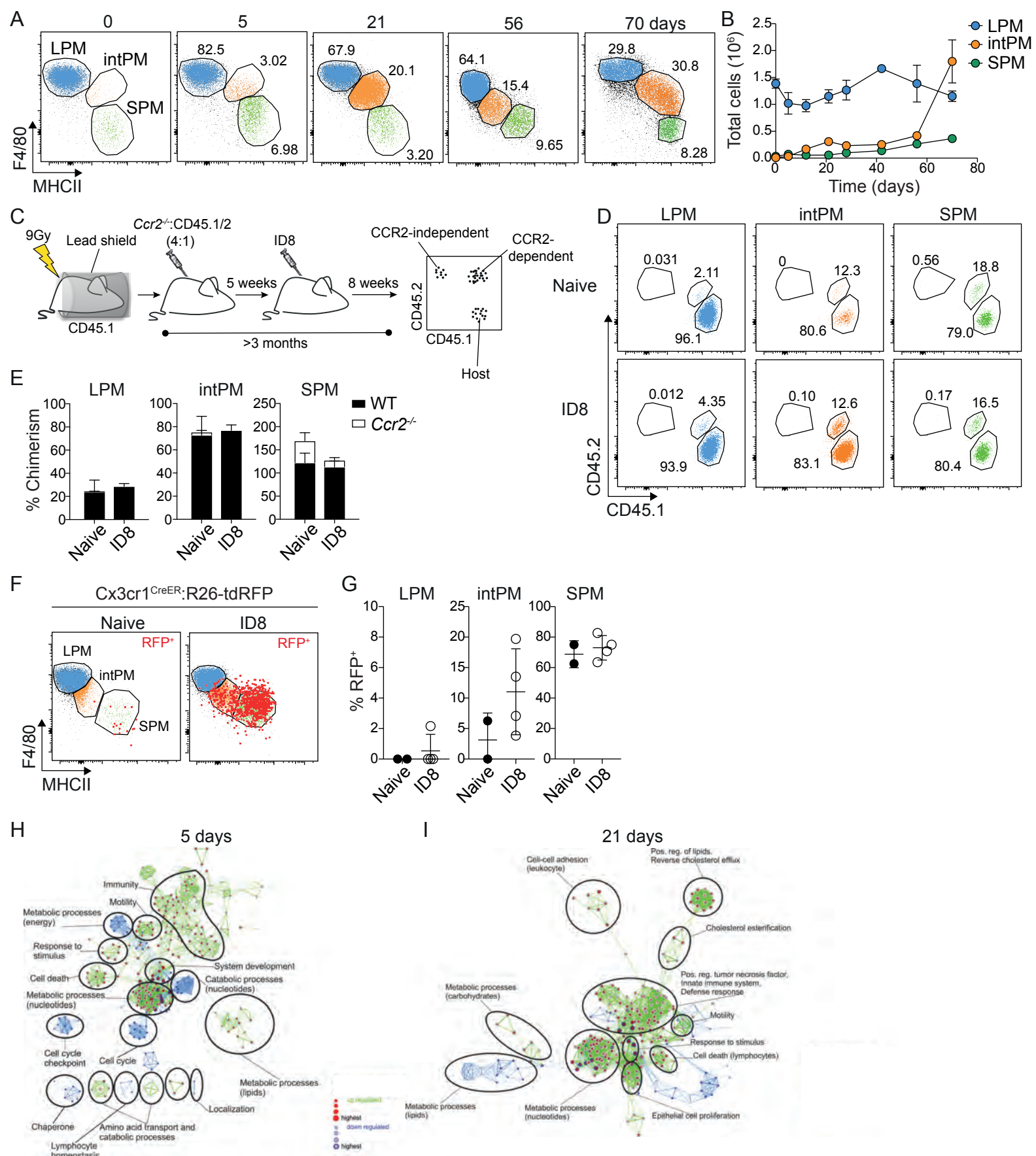


Figure 2

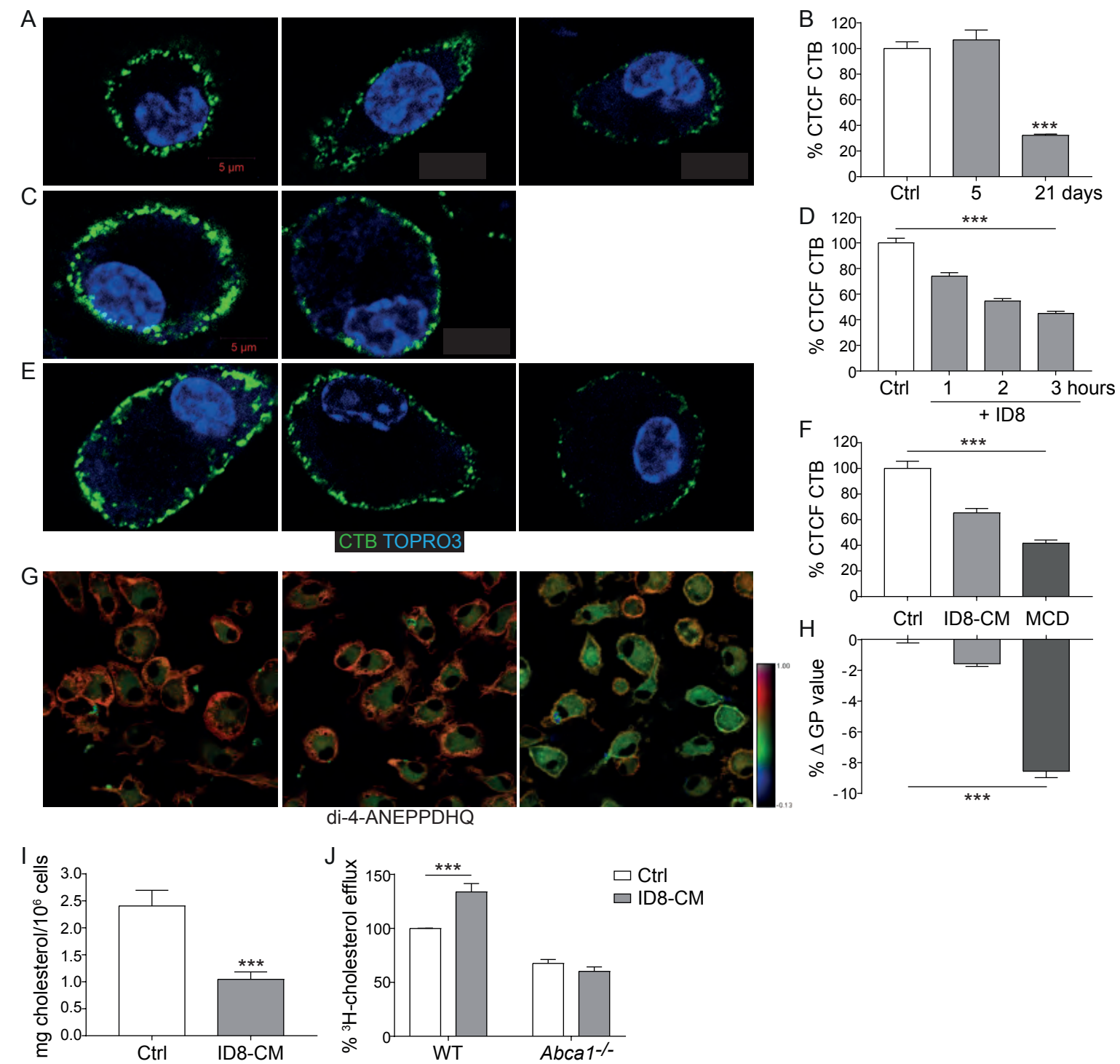


Figure 3

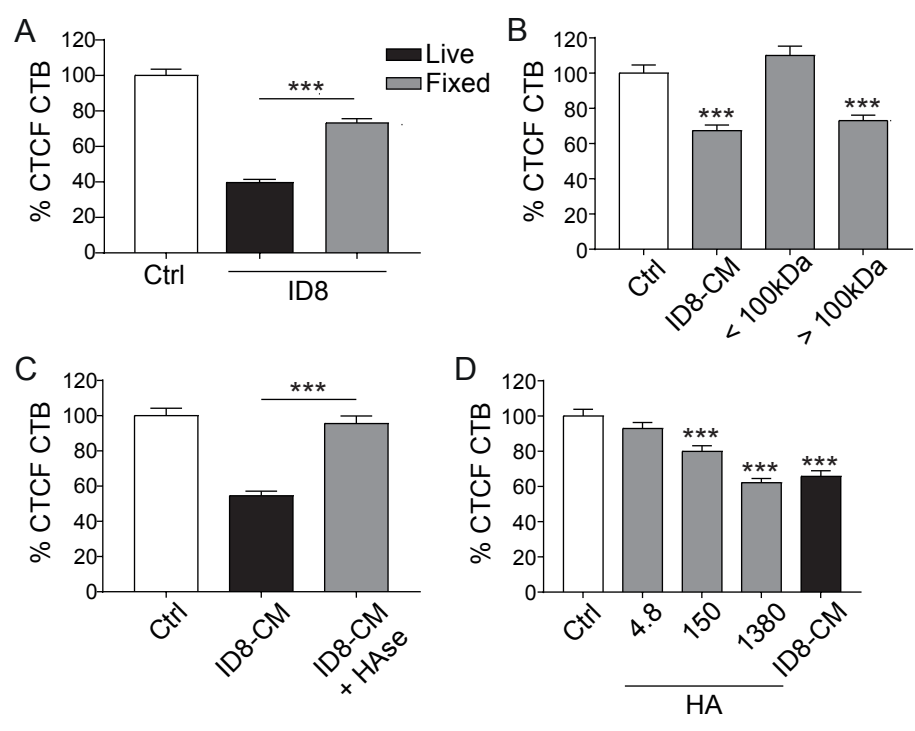
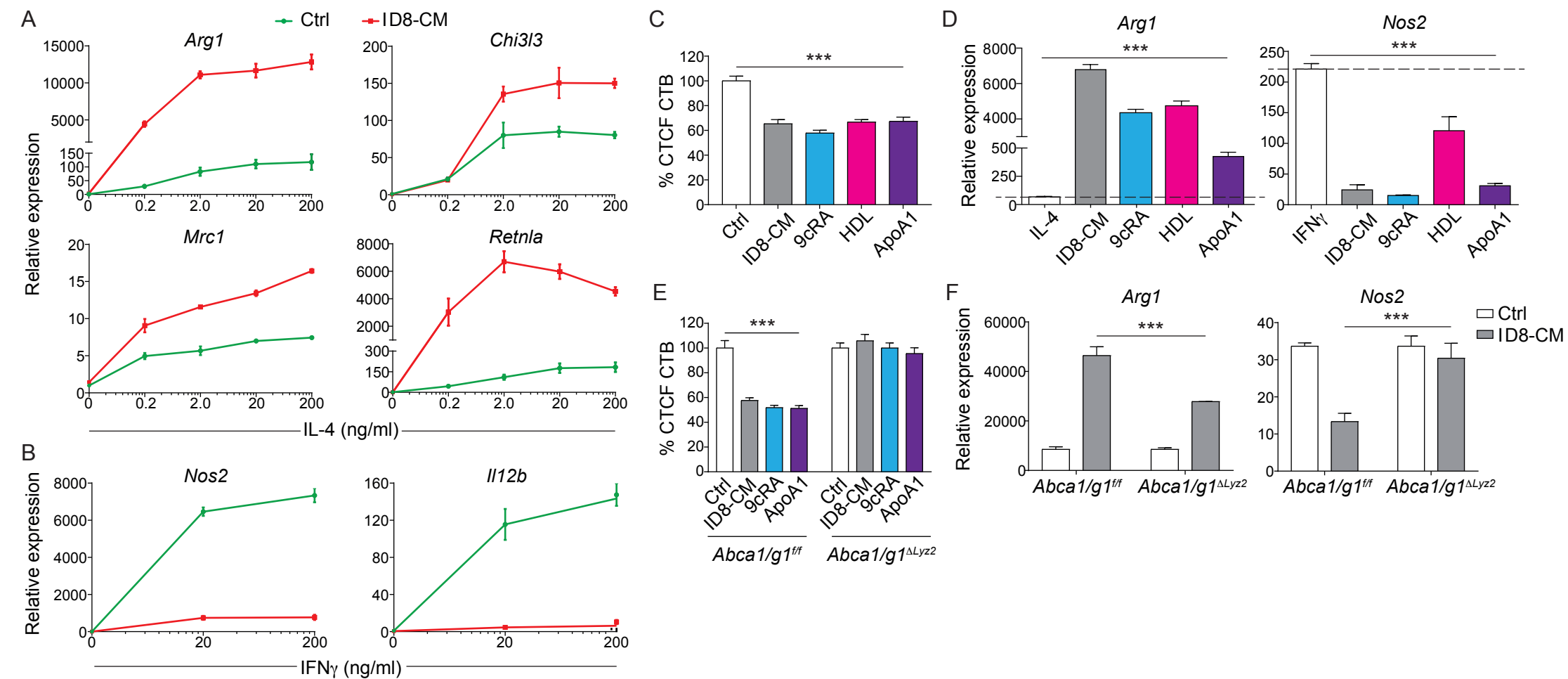


Figure 4





A

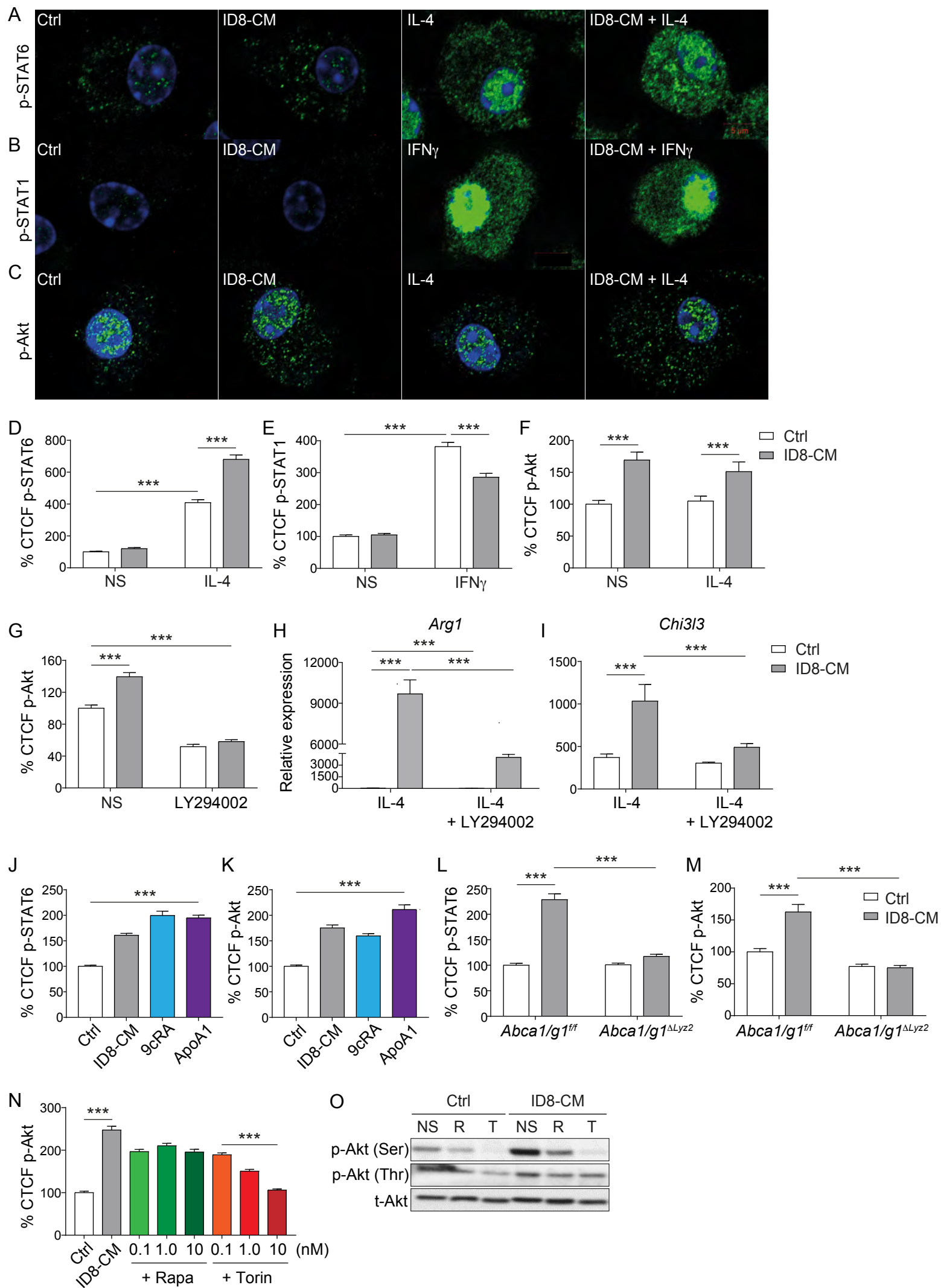
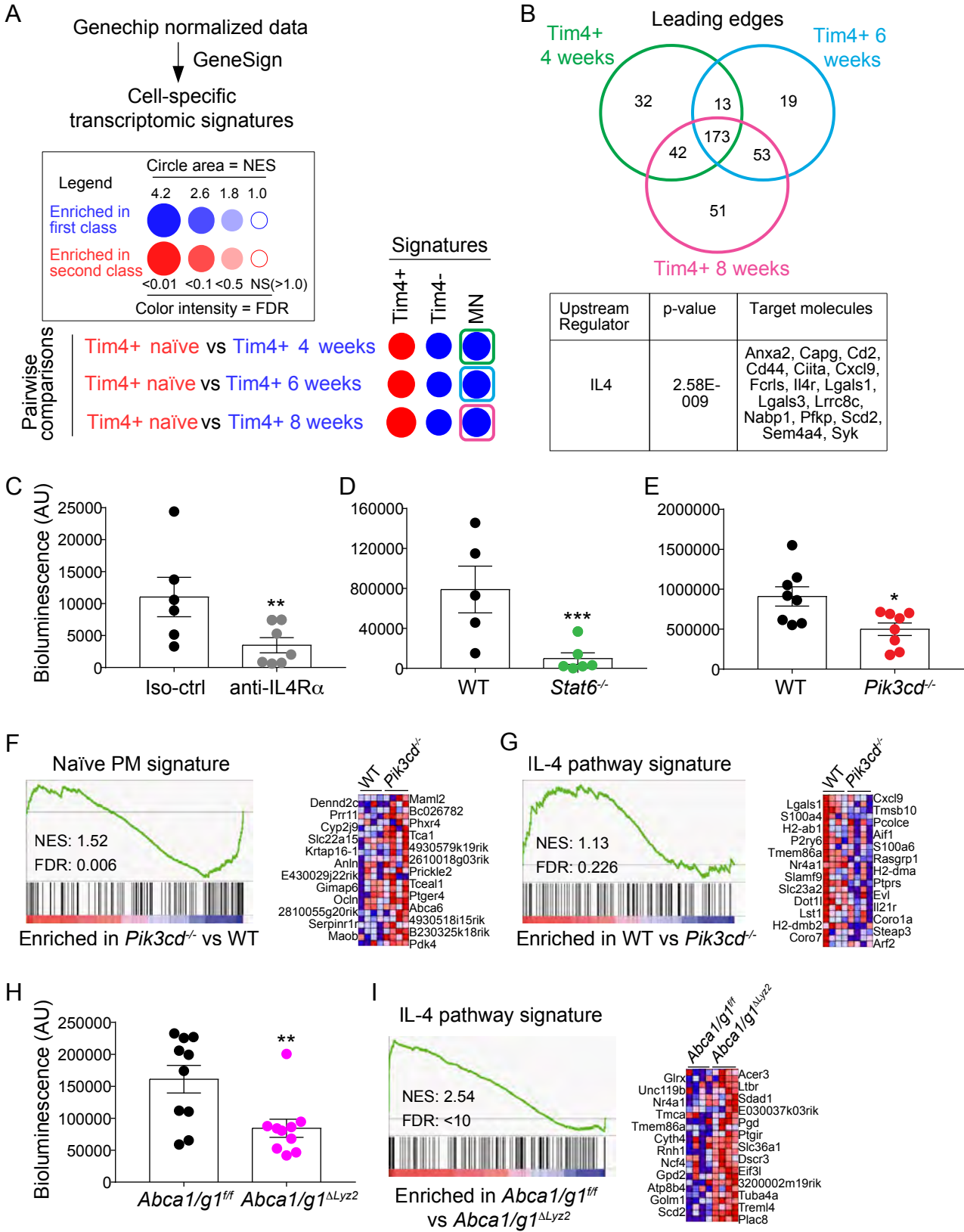


Figure 6



## Supplemental Information

### **Figure S1. CCR2-dependency of peritoneal macrophage subsets and TAM – related to Figure 1.**

Wild-type (WT) and *Ccr2*<sup>-/-</sup> mice were injected with 10<sup>6</sup> ID8-Luc cells i.p. After 8 weeks, mice were euthanized and ascites was harvested by peritoneal lavage and analyzed by flow cytometry. **(A)** Peritoneal macrophages were gated as live cells; CD45.2<sup>+</sup>, Lin<sup>-</sup> (CD5, CD19, Ly6G, NK1.1), CD11b<sup>+</sup> and CD64<sup>+</sup>. Large peritoneal macrophages (LPM) were subsequently gated as F4/80<sup>hi</sup> MHCII<sup>-</sup>, intermediate peritoneal macrophages (intPM) as F4/80<sup>+</sup> MHCII<sup>+</sup> and small peritoneal macrophages (SPM) as F4/80<sup>-</sup> MHCII<sup>+</sup>. **(B)** Total numbers of LPM, intPM and SPM in naïve and ID8-Luc injected mice were calculated. **(C)** Analysis of *Cx3cr1* expression in LPM, intPM and SPM from *Cx3cr1*<sup>egfp/+</sup> reporter mice bearing ID8 tumors. **(D)** Analysis of Lyve-1 expression in LPM, intPM and SPM from ID8-Luc injected mice by flow cytometry.

### **Figure S2. Microarray analysis of TAM versus naïve PM – related to Figure 1.**

**(A)** Gating strategy for sorting of TAM by flow cytometry; bulk TAM were gated as live cells, CD45.2<sup>+</sup>, CD11b<sup>+</sup>, Lin<sup>-</sup> (CD5, CD19, Ly6G, NK1.1), F4/80<sup>+</sup> and CD64<sup>+</sup>. **(B)** Total RNA was extracted from TAM sorted from ascites at 5 (D5), 12 (D12) and 21 days (D21) of tumour growth and naïve peritoneal macrophages (Ctrl). Gene expression was analyzed with MoGene 1.0st microarrays. RMA normalized data were filtered and analyzed for variations in gene expression and hierarchical clustering, the heatmap represents the 1000 most variable genes expressed. **(C)** Sample correlation analysis based on Pearson's correlation coefficients using BioLayout Express3D. **(D)** Ingenuity Pathway Analysis (IPA) with DEGs between naïve PM (Ctrl) and TAM at day 21 (D21). **(E-F)** GSEA comparing TAM with naïve PM using a compiled cholesterol homeostasis geneset; **(E)** Heatmap representing significantly enriched genes in TAM (D21), and **(F)** the enrichment plot indicating normalized enrichment score (NES) and false discovery rate (FDR).

### **Figure S3. ID8 cells promote M2-polarisation of macrophages – related to Figure 4.**

**(A)** BMDM were co-cultured with or without ID8 cells at a 1:1 ratio overnight, ID8 cells were removed by washing before stimulation with IL-4 or IFN $\gamma$  for 8 hours and analysis of *Arg1* and *Nos2* expression by qPCR. **(B)** BMDM were treated with or without ID8-CM before stimulation with IL-13 (20 ng/ml) for 8 hours and *Arg1* expression analysed by qPCR. **(C)** BMDM were stimulated with IL-4 after treatment with fractions of ID8-CM obtained after filtering with cut-offs at 3, 10, 30 and 100 kDa, *Arg1* expression was analysed by qPCR after 8 hours. **(D-E)** BMDM treated with ID8-CM were incubated with and without cholesterol-methyl- $\beta$ -cyclodextrin

(Chl) to replenish membrane cholesterol before stimulation with IL-4 or IFN $\gamma$ ; *Arg1* and *Nos2* expression were analysed by qPCR or western blotting after 8 hours. (F) BMDM were treated with or without ID8-CM before stimulation with IFN $\gamma$  for 8 hours. *Cxcl9*, *Cxcl10* and *Ciita* gene expression was determined by qPCR. Data is represented as mean  $\pm$  SEM of 3 independent experiments.

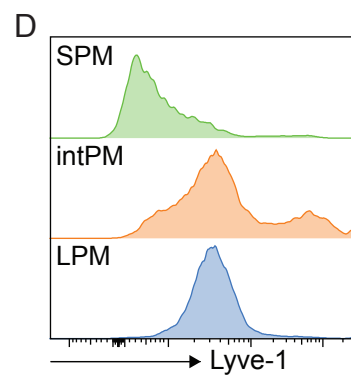
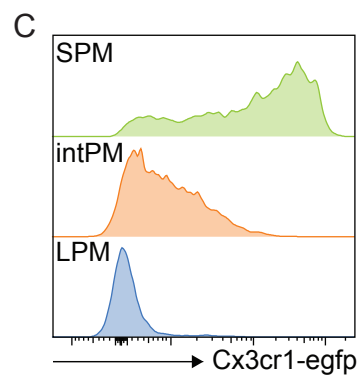
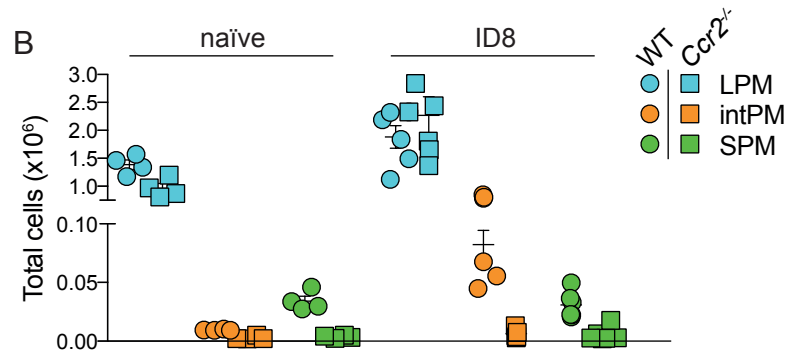
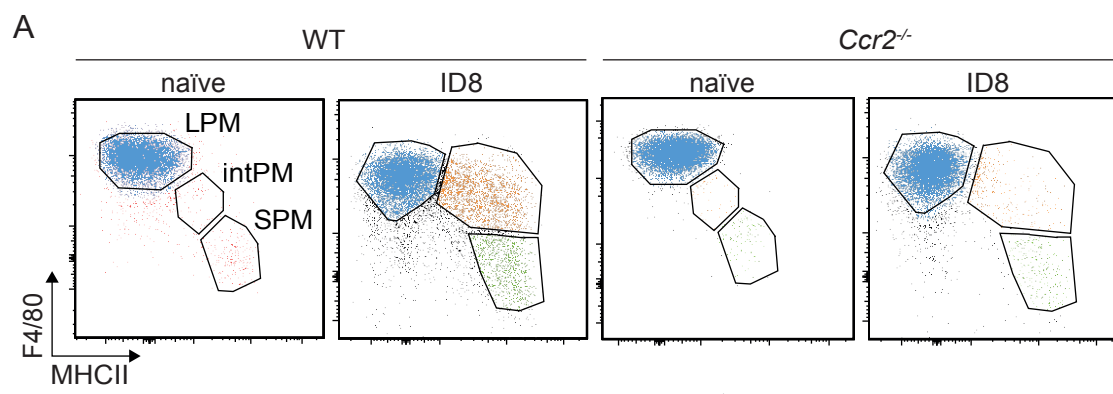
**Figure S4. Tumor-induced macrophage reprogramming is STAT6 and PI3K-dependent – related to Figure 5.**

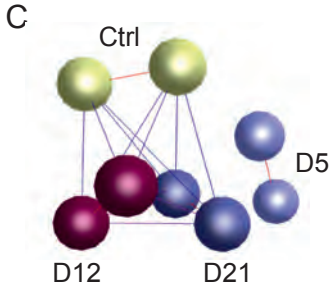
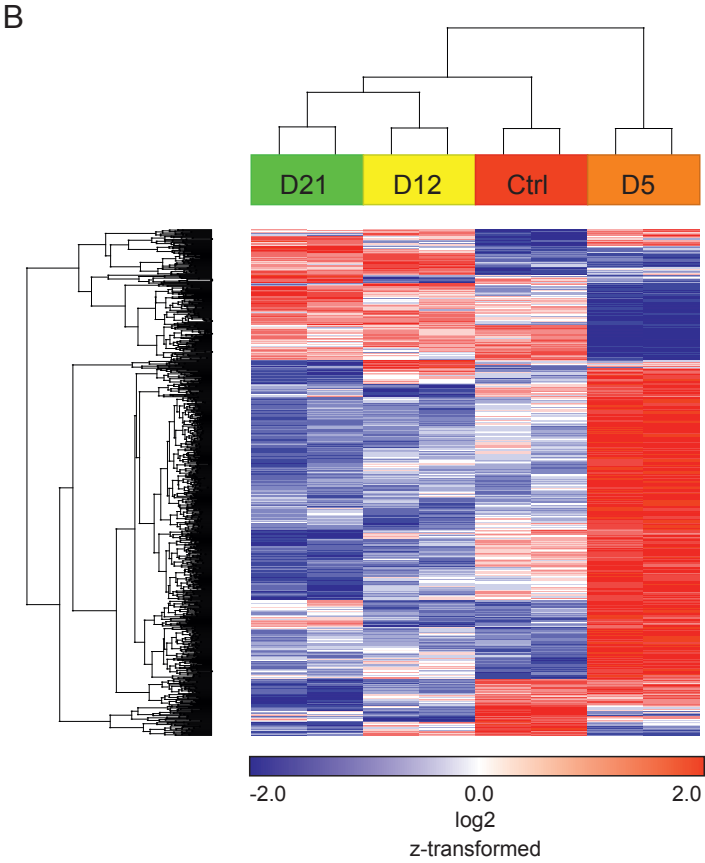
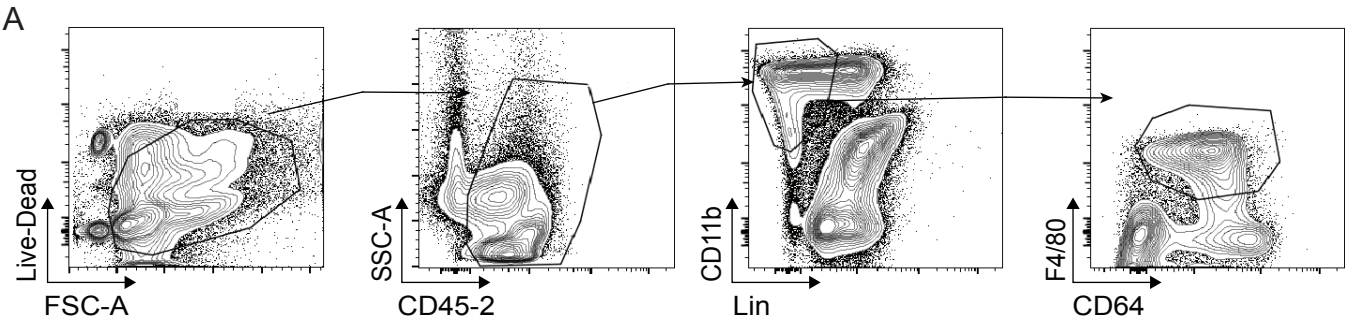
(A,B) BMDM were treated with or without ID8-CM in the absence of cytokine stimulation. After fixation and permeabilization, they were stained for IL-4R and its subcellular location was imaged by confocal microscopy; (A) representative images are shown, (B) receptor clustering was quantified by plotting the mean aggregate size defined as areas larger than 0.2 $\mu$ m<sup>2</sup> with a mean fluorescence intensity higher than twice the background signal. (C-E) BMDM were treated with or without (C) ID8-CM either alone and with subsequent (D) IFN $\gamma$  or (E) IL-4 stimulation; pAKT (ser473), pSTAT1 (Tyr701) and pSTAT6 (Tyr641) were measured by Western Blot at the indicated time points in minutes. Total Akt, STAT1, STAT6 or Actin were used as loading controls. Representative blots from at least 3 independent experiments are shown. (F) BMDM from wild-type (WT) and *Stat6*<sup>-/-</sup> mice were treated with and without ID8-CM before stimulation with IL-4 and *Arg1* expression was measured after 8 hours by western blotting. (G) BMDM were treated with and without ID8-CM before stimulation with IL-4 and accumulation PIP<sub>3</sub> was measured by confocal microscopy. (H) BMDM were incubated with or without ID8-CM in the presence or absence of the PI3K inhibitor LY294002 (1.25  $\mu$ M) before stimulation with IL-4 or IFN $\gamma$ ; *Arg1*, *Retnla*, *Chi3l3* and *Nos2* expression were measured by western blotting after 8 hours. (I) BMDM were stimulated with IL-4 after treatment with fractions of ID8-CM obtained after filtering with cut-off at 100 kDa followed by quantification of p-STAT6 and p-Akt by confocal microscopy. (J) BMDM were incubated with ID8-CM with and without HAse treatment before stimulation with IL-4, p-STAT6 and p-Akt was subsequently measured by confocal microscopy.

**Figure S5. IL-4 signaling and TAM polarization in vivo – related to Figure 6.**

(A) Gating strategy for sorting of monocytes (MN), Tim4<sup>+</sup> and Tim4<sup>-</sup> PM by flow cytometry. (B) Analysis of Tim4<sup>+</sup> and Tim4<sup>-</sup> PM in *Ccr2*<sup>-/-</sup> mice. (C) Reduced expression of TAM marker genes in bulk TAM from *Pik3cd*<sup>-/-</sup> chimeras compared to wild-type (WT) mice at 21 days (D21). (D) GSEA using a tumoricidal gene signature (GSE26912) in naïve PM versus TAM after 21 days (D21). (E) GSEA using the most enriched genes in naïve PM from (D) as a geneset in TAM from WT versus *Pik3cd*<sup>-/-</sup> mice and *Abca1/g1*<sup>ff</sup> versus *Abca1/g1* <sup>$\Delta$ Ly22</sup> mice; enrichment plots are

shown on the left and the 25 most enriched genes on the right. Normalized enrichment score (NES) and false discovery rate (FDR), are indicated. **(F)** Heatmap showing expression of IL-4 responsive genes in TAM from wild-type (WT) and *Stat6*<sup>-/-</sup> mice analysed by high-density quantitative PCR array.





**D**

	p-values	# molecules
<b>Diseases and disorders</b>		
Inflammatory response	2.51E-06 - 1.11E-02	93
Cancer	2.68E-06 - 1.42E-02	205
Immunological disease	2.68E-06 - 1.36E-02	171
<b>Molecular and cellular functions</b>		
Lipid metabolism	9.45E-07 - 1.40E-02	76
Small molecule biochemistry	9.45E-07 - 1.44E-02	105

